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# U.S. PATENT AND TRADEMARK OFFICE
# PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT
under 37 C.F.R. §1.53(b)(2)

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**TITLE OF THE INVENTION (280 characters max)**

Vaccine and Method for Treatment of Neurodegenerative Diseases

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**ENCLOSED APPLICATION PARTS (check all that apply)**

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| [X] Specification | Number of Pages | 70 | [ ]Applicant claims small entity status. See 37 C.F.R. §1.27 |
| [X] Drawing(s) | Number of Sheets | 20 | [ ] Other (specify) _____ |

**METHOD OF PAYMENT (check one)**

[X] Credit Card Payment Form PTO-2038 is enclosed to cover the Provisional filing fee of
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[X] The Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number 02-4035

The invention was made by an agency of the United Stated Government or under a contract with an agency of the United States Government.

[X] No [ ] Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

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13281 U.S. PTO



# VACCINE AND METHOD FOR TREATMENT OF NEURODEGENERATIVE DISEASES

## FIELD OF THE INVENTION

The present invention relates to compositions, e.g. vaccines, and methods for the treatment of neurodegenerative diseases in which there is accumulation of misfolded and/or aggregated proteins, excluding prion diseases. In particular, the invention relates to treatment of the neurodegenerative diseases Huntington's disease (HD), Alzheimer's disease (AD) or Parkinson's disease (PD), by administration of an agent selected from the group consisting of Copolymer 1, a Copolymer 1-related peptide or polypeptide, or T cells activated therewith.

**ABBREVIATIONS I: AD**, Alzheimer's disease; **$A\beta_{1-40}$**, β-amyloid$_{1-40}$; **CNS**, central nervous system; **Cop-1**, Copolymer 1; **HD**, Huntington's disease; **IRPB**, interphotoreceptor retinoid-binding protein; **OHSC**, organotypic hippocampal slice culture; **PD**, Parkinson's disease; **PI**, propidium iodide; **RGC**, retinal ganglion cell; **Treg**, $CD4^+CD25^+$ regulatory T cells; **TUNEL**, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling; **WRH**, whole retinal homogenate.

## BACKGROUND OF THE INVENTION

**~~Diseases involving~~ accumulation of misfolded and/or aggregated proteins**

For many decades, clinicians have been aware of the formation of insoluble protein aggregates in particular diseases. In Alzheimer disease (see Selkoe, D.J. 2002. Deciphering the genesis and fate of amyloid-β protein yields novel therapies for Alzheimer disease. *J. Clin. Invest.* In press), the presence in the CNS of β-amyloid–containing plaques is associated with neurodegeneration and dementia (Selkoe, D. 1997. Cellular and molecular biology of the beta-amyloid precursor

protein and Alzheimer's disease. In *The molecular and genetic basis of neurological disease.* R.N. Rosenberg, S.B. Prusiner, S. DiMauro, and R.L. Barchi, editors. Butterworth-Heinemann. Boston, Massachusetts, USA. 601–611). Similarly, other neurodegenerative diseases have recently been discovered to involve protein aggregation. For example, prion diseases such as Creutzfeldt-Jacob disease and bovine spongiform encephalopathy are associated with amyloid deposits of the PrP protein (DeArmond, S.J., and Prusiner, S.B. 1997. Molecular neuropathology of prion diseases. In *The molecular and genetic basis of neurological disease.* R.N. Rosenberg, S.B. Prusiner, S. DiMauro, and R.L. Barchi, editors). Polyglutamine repeat diseases such as Huntington disease are likewise associated with neuronal cytosolic and intranuclear inclusions (DiFiglia, M. *et al.* (1997). Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. *Science.* **277**, 1990–1993). These inclusions are composed of fibrils that stain similarly to amyloid (Scherzinger, E. *et al.* (1997). Huntintin-encoded polyglutamine expansions form amyloid-like protein aggregates in vitro and in vivo. *Cell.* **90**, 549–558). Finally, in Parkinson disease, inclusions known as Lewy bodies, found in the cytoplasm of cells of the basal ganglia, include amyloid-like aggregates of the protein α-synuclein (Conway, K.A., Harper, J.D., & Lansbury, P.T. (2000). Fibrils formed in vitro from α-synuclein and two mutant forms linked to Parkinson's disease are typical amyloid. *Biochemistry.* **39**, 2552–2563; Serpell, L.C., Berriman, J., Jakes, R., Goedert, M., & Crowther, R.A. (2000). Fiber diffraction of synthetic α-synuclein filaments shows amyloid-like cross-β conformation. *Proc. Natl. Acad. Sci. USA.* **97**, 4897–4902).

Alzheimer's disease (~~AD~~) ~~is an irreversible,~~ progressive brain disorder that occurs gradually and results in memory loss, behavioural and personality changes, and a decline in mental abilities. These losses are related to the death of brain cells and the breakdown of the connections between them. The course of this disease varies from person to person, as does the rate of decline. On average, AD patients live for 8 to 10 years after they are diagnosed, though the disease can last up to 20 years.

AD advances by stages, from early, mild forgetfulness to a severe loss of mental function. At first, AD destroys neurons in parts of the brain that control memory, especially in the hippocampus and related structures. As nerve cells in the hippocampus stop functioning properly, short-term memory fails. AD also attacks the cerebral cortex, particularly the areas responsible for language and reasoning. Eventually, many other areas of the brain are involved.

There are important parallels between AD and other neurological diseases, including prion diseases (such as kuru, Creutzfeld-Jacob disease and bovine spongiform encephalitis), Parkinson's disease, Huntington's disease, and fronto-temporal dementia. All involve deposits of abnormal proteins in the brain. AD and prion diseases cause dementia and death, and both are associated with the formation of insoluble amyloid fibrils, but from membrane proteins that are different from each other.

**Protective autoimmunity**

Loss of neurons in patients with devastating chronic neurodegenerative disorders is attributed to numerous factors, most of them (for example, oxidative stress, ion imbalance, metabolic deficits, neurotransmitter imbalance, neurotoxicity) common to all such diseases [1]. Even those factors that are apparently unique to a particular disorder share certain common features, including changes in the extracellular deposition of self-compounds resulting in conformational and other changes, as well as in their aggregation, often culminating in plaque formation [2].

The local immune response to injuries in the central nervous system (CNS) has often been blamed for the progressive neurodegeneration that occurs after an insult [3]. Studies in the inventors' laboratory, however, have challenged the long-held notion that activated microglia or blood-borne activated macrophages contribute to the ongoing pathology, and suggest instead that these immune cells are harnessed to aid recovery, but may be unable to display a significant positive effect because they fail to acquire the necessary phenotype (activity) or because their intervention is not strong enough or is inappropriately timed. This suggestion was supported by the demonstration that, in rats, macrophages activated by peripheral

nerve [4] or by skin [5] can be helpful in promoting recovery from spinal cord injury. The functional activity of such macrophages was recently found to resemble that of APC [5].

Subsequent studies by the inventors suggested that after a mechanical or biochemical insult to the CNS the local immune response, which is mediated by T cells directed against self-antigens residing in the site of the lesion (i.e., autoimmune T cells), determines the ability of the neural tissue to withstand the unfriendly extracellular conditions resulting from the injury. It thus seems that the body protects itself against toxic self-compounds in the CNS by harnessing a peripheral adaptive immune response in the form of T cells specific to antigens residing in the site of damage [6-10]. The T cells that mediate protection are directed not against a particular threatening self-compound but rather against dominant self-antigens that reside at the lesion site [11-13].

Further studies by the inventors suggested that T-cell specificity is needed in order to ensure that among the T cells that arrive at the site, those encountering their specific or cross-reactive antigens (presented to them by local microglia acting as APC) will become activated. The activated T cells can then provide the necessary cytokines or growth factors that control the activity of the local microglia and the friendliness of the extracellular milieu [12, 14, 15].

The concept of T cell-dependent "protective autoimmunity" has been formulated by the inventor Prof. Michal Schwartz and her group [25, 26]. According to this concept, an acute or chronic insult to the CNS triggers an autoimmune response directed against proteins residing in the lesion site. T cells homing to the lesion site are activated by ~~cells presenting~~ the relevant antigen. Once activated, they augment and control local immune cells, allowing efficient removal of toxic compounds and tissue debris, thus protecting the damaged nerves from further degeneration. The potential of the immune system to counteract the hostile conditions is enhanced by boosting the normal immune response. Based on this hypothesis, boosting the immune system with a suitable antigen should provide

neuroprotection. Among suitable antigens identified by the present inventors is Copolymer 1.

Copolymer 1 (Cop 1 or Glatiramer) is a random non-pathogenic synthetic copolymer composed of the four amino acids, L-tyrosine, L-glutamate, L-lysine and L-alanine. Glatiramer acetate has been approved in several countries for the treatment of multiple sclerosis under the trademark Copaxone® (a trademark of Teva Pharmaceutical Industries Ltd., Petach Tikva, Israel).

Vaccination with Cop 1 or with Cop 1-activated T cells have been shown by the present inventors to boost the protective autoimmunity, after traumatic CNS insult, thereby reducing further injury-induced damage, and can further protect CNS cells from glutamate toxicity. Reference is made to our previous United States Patent Application Serial Nos. 09/756,301 and 09/765,644, both dated 22 January, 2001, corresponding to WO 01/93893, which disclose that Cop 1, Cop 1-related peptides and polypeptides and T cells activated therewith protect CNS cells from glutamate toxicity (USSN 09/756,301) and prevent or inhibit neuronal degeneration or promote nerve regeneration in the CNS or PNS (USSN 09/765,644; WO 01/52878).

Prof. Schwartz and colleagues have shown that Cop 1 acts as a low-affinity antigen that activates a wide range of self-reacting T cells, resulting in neuroprotective autoimmunity that is effective against both CNS white matter and grey matter degeneration [25, 26]. The neuroprotective effect of Cop 1 vaccination was demonstrated by the inventors in animal models of acute and chronic neurological disorders such as optic nerve injury (15), head trauma (Kipnis et al., 2003), glaucoma (9), amyotrophic lateral sclerosis (30) and in the applicant's patent applications WO 01/52878, WO 01/93893 and WO 03/047500.

US Provisional Application No. 60/518, 627 of the present applicants, filed November 12, 2003, discloses Cop 1 vaccination for the treatment of Huntington's disease.

WO 01/97785 discloses the use of Cop 1 for treating prion-related diseases.

All patents and patent applications cited herein are hereby incorporated by reference in their entirety as if fully disclosed herein.

## SUMMARY OF THE INVENTION

The present invention relates, in one aspect, to a method for treating a neurodegenerative disorder or disease in which there is accumulation of misfolded and/or aggregated proteins, excluding prion-related diseases, said method comprising administering to an individual in need an agent selected from the group consisting of (i) Copolymer 1, (ii) a Copolymer 1-related peptide, (iii) a Copolymer 1-related polypeptide, and (iv) T cells activated with (i), (ii) or (iii).

In one embodiment, the invention relates to a method for reducing disease progression, and/or protection from neurodegeneration and/or protection from glutamate toxicity in a patient suffering from a neurodegenerative disorder or disease in which there is accumulation of misfolded and/or aggregated proteins, excluding prion-related diseases, which comprises administering to said patient a therapeutically active amount of an agent selected from the group consisting of (i) Copolymer 1, (ii) a Copolymer 1-related peptide, (iii) a Copolymer 1-related polypeptide, and (iv) T cells activated with (i), (ii) or (iii).

In another embodiment, the invention relates to a method for reducing disease progression, and/or protection from neurodegeneration and/or protection from glutamate toxicity in a patient suffering from a neurodegenerative disorder or disease in which there is accumulation of misfolded and/or aggregated proteins, excluding prion-related diseases, which comprises immunizing said patient with an agent selected from the group consisting of (i) Copolymer 1, (ii) a Copolymer 1-related peptide, (iii) a Copolymer 1-related polypeptide, and (iv) T cells activated with (i), (ii) or (iii).

In another aspect, the present invention provides a pharmaceutical composition for treatment of a neurodegenerative disorder or disease in which there is accumulation of misfolded and/or aggregated proteins, excluding prion-related diseases, comprising a pharmaceutically acceptable carrier and an active agent selected from the group consisting of (i) Copolymer 1, (ii) a Copolymer 1-related

peptide, (iii) a Copolymer 1-related polypeptide, and (iv) T cells activated with (i), (ii) or (iii). In one embodiment, said pharmaceutical composition is a vaccine.

In a further aspect, the present invention relates to the use of an active agent selected from the group consisting of (i) Copolymer 1, (ii) a Copolymer 1-related peptide, (iii) a Copolymer 1-related polypeptide, and (iv) T cells activated with (i), (ii) or (iii), for the manufacture of a pharmaceutical composition for treatment of a neurodegenerative disorder or disease in which there is accumulation of misfolded and/or aggregated proteins, excluding prion-related diseases. In one embodiment, said pharmaceutical composition is a vaccine.

In one embodiment, said neurodegenerative disease or disorder is Huntington's disease. In another embodiment, said neurodegenerative disease or disorder is Alzheimer's disease. In a further embodiment, said neurodegenerative disease or disorder is Parkinson's disease.

In the most preferred embodiment of the invention, the active agent is Copolymer 1.

## BRIEF DESCRIPTION OF THE FIGURES

**Figs. 1A-1D** show that immunization of mice with retinal proteins protects retinal ganglion cells against glutamate toxicity. (A) TUNEL-positive cells in the RGC layers of C57Bl/6J mice 48 h after intravitreal injection of a toxic dose of glutamate. Sections (20 μm thick) were subjected to TUNEL staining, counterstained with propidium iodide, and viewed by confocal microscopy to detect TUNEL-positive cells. A confocal image of a representative retina is shown. The arrow indicates TUNEL-positive cells in the RGC layer. Scale bar = 200 μm. (B) C57Bl/6J mice were immunized in the flank with 600 μg of whole retinal homogenate (WRH) emulsified in CFA supplemented with 5 mg/ml of *Mycobacterium tuberculosis*. Six days later the mice were injected intravitreally with glutamate (400 nmol). One week later surviving RGCs were counted. Significantly more RGCs survived in mice immunized with WRH/CFA than in mice immunized with PBS/CFA. The figure shows the results, expressed as

neuronal loss relative to the RGC population of normal retinas, of one representative experiment out of two independent experiments (n = 6–8 mice per experiment in each group; $P < 0.0001$, two-tailed Student's *t*-test). (C, D) In another set of experiments mice were immunized in the flank with interphotoreceptor-binding protein (IRBP; 50 μg) or S-antigen (50 μg) emulsified in CFA supplemented with 5 mg/ml of *Mycobacterium tuberculosis*. Control mice were immunized with PBS in CFA. Significantly more neurons were lost in the PBS/CFA immunized group than in the IRBP/CFA immunized group ($P < 0.0001$; two-tailed Student's *t*-test; n = 6–8 mice in each group) or in the S-antigen-immunized group ($P < 0.0001$; n = 6–8 mice per group).

**Figs. 2A-2F** show that susceptibility of retinal ganglion cells to $A\beta_{1-40}$ toxicity is T cell-dependent. (A) C57BL/6J mice were injected intravitreally with 5 or 50 μM $A\beta_{1-40}$ or were not injected (control), and 1 or 2 weeks later the retinas were excised and the surviving RGCs counted. Relative to controls, significantly more neurons were lost in mice that had received the higher dose of $A\beta_{1-40}$ ($P < 0.001$ and $P < 0.0001$ at 1 and 2 weeks, respectively, two-tailed Student's *t*-test). Values shown are from one of three independent experiments with similar results (n = 6–8 mice per group). Injection of the vehicle resulted in a small loss of RGCs relative to controls. (B) and (C), representative micrographs of retinas injected or not injected with $A\beta_{1-40}$. (D) BALB/c/OLA mice [wild type or nu/nu (devoid of mature T cells)] were injected intravitreally with $A\beta_{1-40}$ (50 μM). Significantly more RGCs were lost in the nu/nu mice relative to the wild-type ($P < 0.001$, two-tailed Student's *t*-test) Values shown are from one of three independent experiments with similar results (n = 5–7 mice per group). (E) and (F), representative micrographs of retinas from wild-type BALB/c/OLA and nu/nu BALB/c/OLA mice injected with $A\beta_{1-40}$.

**Figs. 3A-3B** show that immunization with an antigen residing in the site of toxicity rather than with the toxic agent itself protects against aggregated $A\beta_{1-40}$ toxicity in C57Bl/6J mice. C57Bl/6J mice were immunized in the flank with

interphotoreceptor-binding protein (IRBP; 50 μg) in CFA, the β-amyloid peptide (1–40, non-aggregated) (50 μg) in CFA, or PBS in CFA. In all cases, CFA was supplemented with 5 mg/ml of *Mycobacterium tuberculosis*. Ten days later the mice were injected intravitreally with a toxic dose of aggregated $A\beta_{1-40}$ (50 μM), and after 10 days their retinas were excised and the surviving RGCs counted. (A) Significantly fewer RGCs were lost in C57BL6/J mice immunized with IRBP/CFA than in matched controls treated with PBS/CFA ($P < 0.0008$, two-tailed Student's *t*-test). (B) The mean number of surviving RGCs in mice immunized with native β-amyloid peptide in CFA did not differ significantly from that in mice injected with PBS/CFA.

**Figs. 4A-4B** show that passive transfer of activated splenocytes from mice immunized with dominant retinal antigens into naïve mice results in protection. *(A)* Wild-type C57Bl/6J mice were immunized in the hind foot pads with a combination of interphotoreceptor-binding protein (IRBP) and S-antigen (50 μg each) or 50 μg OVA emulsified in CFA supplemented with 5 mg/ml of *Mycobacterium tuberculosis*. Ten days later draining lymph nodes were excised and pooled, cell suspensions were prepared, and the cells were counted. Cells were activated ex-vivo by stimulation with their specific antigens for 48 h, and the activated T cells were then injected i.p. into naïve C57Bl/6J mice. T cells specific to IRBP + S-antigen were injected at a dose of $1.2 \times 10^7$ T cells in PBS. Within 1 h of passive T cell transfer the mice received an intravitreal injection of glutamate (400 nmol), and surviving retinal ganglion cells (RGCs) were counted 1 week later. Significantly fewer RGCs survived in mice that received OVA-specific T cells than in mice that received T cells specific to IRBP + S-antigen ($P < 0.001$; two-tailed Student's *t*-test). There was no difference between mice that received OVA-specific T cells and naïve mice in the numbers of RGCs that survived the glutamate injection (n = 4–6 mice per group). (B) Mice were injected intravenously with $8\times10^6$ activated T cells directed either to IRBP or to β-amyloid peptide (1–40, non-aggregated). One hour after this passive T-cell transfer, the mice were injected with a toxic dose of

aggregated $A\beta_{1-40}$. Two weeks later their retinas were excised and surviving RGCs counted. Neuronal loss in these mice was significantly decreased by transfer of T cells reactive to the IRBP ($P < 0.005$, two-tailed Student's *t*-test), but was not significantly affected by transfer of T cells reactive to non-aggregated β-amyloid.

**Fig. 5** shows that active immunization with Cop-1 protects against $A\beta_{1-40}$ toxicity. C57Bl/6J mice were immunized with Cop-1, 6 days before being injected intravitreally with aggregated $A\beta_{1-40}$. Two weeks later their retinas were excised and the surviving cells counted. Significantly fewer RGCs were lost in mice immunized with Cop-1 than in matched controls treated with PBS ($P < 0.001$, two-tailed Student's *t*-test).

**Figs. 6A-6C** show that more neurons survive aggregated $A\beta_{1-40}$ intoxication in mice devoid of naturally occurring regulatory CD4+CD25+ T cells than in naïve mice. (A) C57Bl/6J mice devoid of Treg as a result of thymectomy 3 days after birth (TXD3 mice) were injected intravitreally with a toxic dose of $A\beta_{1-40}$ at the age of 12 weeks. Significantly fewer RGCs were lost in the TXD3 mice than in age-matched normal controls ($P < 0.001$; two-tailed Student's t-test; $n = 6–8$ mice per group). (B) BALB/c/OLA nu/nu mice were replenished with $4.5 \times 10^7$ splenocytes from spleens devoid of Treg or from whole spleens of BALB/c/OLA mice. After injection of aggregated $A\beta_{1-40}$, significantly fewer RGCs were lost in nu/nu mice replenished with splenocytes devoid of Treg than in matched wild-type controls ($P < 0.05$; two-tailed Student's t-test). In both groups, significantly fewer RGCs were lost than in untreated nu/nu mice injected with aggregated $A\beta_{1-40}$ ($P < 0.001$; two-tailed Student's t-test). In each experiment, the number of RGCs counted in eyes not exposed to aggregated $A\beta_{1-40}$ toxicity was taken as the normal baseline value. The results of one experiment out of two are presented. (C) Semi-quantitative RT–PCR analysis of Foxp3 expression. mRNA was extracted from freshly isolated Teff and Treg. The housekeeping gene β-actin was used for quantitative analysis. The results shown are from one representative experiment out of five.

**Figs. 7A-7B** show death of neural cells in rat organotypic hippocampal slice cultures 24 h after treatment with microglia incubated with aggregated $A\beta_{1-40}$ with and without activated T cells. OHSCs were obtained from BALB/c/OLA mice. Immediately after sectioning, the slices were co-cultured for 24 h with microglia that had been pre-incubated (12 h) with aggregated $A\beta_{1-40}$ alone or with a combination of aggregated $A\beta_{1-40}$ and activated Teff (A). Control slices were treated with naïve microglia or were left untreated. Twenty-four hours after co-culturing of microglia and brain slices, the slices were stained with propidium iodide (PI) (a fluorescent dye that stains only dead cells) and analyzed by fluorescence microscopy. (A) quantification of PI intensity, calculated as a percentage of the intensity measured in untreated control OHSCs (*$P < 0.05$; ** $P < 0.01$, *** $P < 0.001$; two-tailed Student's t-test; n = 6–8 slices per group). (B), selected photomicrographs of untreated control slices (1), slices incubated with untreated microglia (2), slices treated with microglia that were pre-incubated with aggregated $A\beta_{1-40}$ (3), and slices treated with microglia that had been exposed to aggregated $A\beta_{1-40}$ in conjunction with activated T cells (4).

**Figs. 8A-8C** show that aggregated β-amyloid confers a cytotoxic phenotype on microglia. (A) Organotypic hippocampal slices (OHSCs) were co-cultured with microglia (MG) that were pre-incubated for 24 h with aggregated β-amyloid ($MG_{A\beta(1-40)}$) or LPS ($MG_{LPS}$). Control microglia ($MG_{(-)}$)were left untreated. As controls for cytotoxicity or protection we used slices free of microglia. After 24 h of co-culturing the slices were washed, stained with PI, and analyzed by confocal microscopy. PI staining (red) indicates cell death in OHSCs. (B) Statistical analysis shows intensity of PI staining, calculated as a percentage of the intensity measured in untreated control OHSCs (means ± SD; two-tailed Student's *t*-test; $n = 8–10$ slices per group, obtained in two independent experiments). *, $P < 0.05$; ***, $P < 0.001$. (C) Expression of Ii, CIITA, STAT1, and TNF-α mRNA in microglia treated for 12 h with LPS (100 ng/ml) or $A\beta_{(1-40)}$; (50 μM) by semi-quantitative RT–PCR. Control microglia were incubated with medium alone (Cntr). Note the increase in

TNF-α transcripts in the $MG_{LPS}$ and $MG_{A\beta(1-40)}$ relative to control microglia. Values represent the relative amounts of amplified mRNA normalized against β-actin (β-act) in the same sample, and are expressed as fold of induction relative to control (means ± SD). The means of three independent experiments are shown.

**Figs. 9A-9C** show that IL-4 and IFN-γ endow microglia with a neuroprotective phenotype. Microglia were treated with IFN-γ or IL-4 and then co-cultured with hippocampal slices. Neural cell death in OHSCs was determined 24 h after their exposure to microglia pretreated for 12 h or 24 h with different concentrations of IL-4 or IFN-γ. Note that the maximum neuroprotective effect was observed with IL-4-treated microglia ($MG_{IL-4}$; 24 h). Microglia treated with IFN-γ ($MG_{IFN-\gamma}$) showed a slight neuroprotective effect at lower concentrations and were cytotoxic at the highest concentration. Results are expressed by the percentage of PI staining intensity relative to untreated OHSCs (means ± SD). Statistical significance was analyzed by two-tailed Student's *t*-test ($n$ = 8–10 slices per group). Note, asterisks above each bar express the difference relative to untreated control slices ($^{*}$, $P < 0.05$; $^{**}$, $P < 0.01$; $^{***}$, $P < 0.001$). (B) Expression of Ii, CIITA, STAT1, and TNF-α mRNA in microglia incubated for 12 h with medium alone (Cntr) or treated for 12 h with IFN-γ (20 ng/ml) or IL-4 (10 ng/ml). Values represent the relative amounts of amplified mRNA measured by PCR amplification and normalized against β-actin in the same sample, and are expressed as fold induction relative to control (means ± SD). Representative results of one of three independent experiments are shown. (C) IL-4 and IFN-γ time courses of TNF-α and CIITA mRNA expression in cultured microglia. PCR at each time point was performed from the same reverse transcription mixtures for all cDNA species. The linear working range of amplifications was ascertained prior to experiments. The figure shows one of two independent experiments carried out with two different microglial cultures.

**Figs. 10A-10C** show time course of induction of MHC-II expression in microglia activated by LPS, $A\beta_{(1-40)}$, IFN-γ, or IL-4. (A) Microglia were treated

with LPS (100 ng/ml), $A\beta_{(1-40)}$ (50 μM), IFN-γ (20 ng/ml), or IL-4 (10 ng/ml) for 12 h or 24 h. Untreated microglial cells were used as controls. Confocal images represent microglia, identified by staining for isolectin B4 (I-B4, green), MHC-II (red), and nuclei (PI, blue) after 24 h of treatment. Untreated microglia did not express MHC-II. LPS and $A\beta_{(1-40)}$ caused microglia to change their shape (to an ameboid form) but failed to induce MHC-II expression. After exposure to IFN-γ or IL-4 the microglia expressed MHC-II. Cytokine concentrations were chosen according to the maximal protection provided, as shown in Figure 2A. (B) Expression of MHC-II was analyzed after treatment for 12 h and 24 h. Values are MHC-II fluorescence intensity per cell, calculated as a percentage of the intensity measured in control (defined as 100%; means ± SD; obtained in three independent experiments, each repeated four times). Asterisks above each bar express the significance of the difference relative to control ($^{**}$, $P < 0.01$; $^{***}$, $P < 0.001$; two-tailed Student's *t*-test). Groups were compared by ANOVA and significant differences between groups are expressed as *P* values. (C) Quantification of apoptotic cells in $MG_{LPS}$ (100 ng/ml) or $MG_{A\beta(1-40)}$ (50 μM) for 24 h. Microglia, identified by staining for ED1, were stained for apoptosis by TUNEL and for nuclei by PI. Some apoptosis occurred during the 24-h treatment of microglia with LPS (100 ng/ml), but did not differ significantly from that seen in control and was not co-localized with ED1-positive cells.

**Figs. 11A-11F** show that aggregated β-amyloid and LPS affect the microglial phenotype in a way that cannot be reversed by IFN-γ and is partially reversible by IL-4. (A) Down-regulation of Ii and CIITA transcripts and increase in TNF-α transcripts in $MG_{LPS}$ or $MG_{A\beta(1-40)}$. Microglia were stimulated with the indicated sequential treatments of LPS-and IFN-γ or $A\beta_{(1-40)}$ and IFN-γ, each for 12 h. Total RNA was isolated and analyzed for Ii, CIITA, STAT1, and TNF-α mRNA by semi-quantitative RT–PCR. (B) Values represent the relative amounts of amplified mRNA normalized against β-actin in the same sample, and are expressed as fold of induction relative to untreated microglia (means ± SD). The means of

three independent experiments are shown. (C) Quantitative real-time PCR (Q-PCR) revealed a significant decrease in Ii and CIITA transcripts of $MG_{A\beta(1-40)}$ treated with IFN-γ compared to ($MG_{IFN-\gamma}$). (D–F) Quantitative analysis of cell-surface expression of MHC-II analyzed by confocal microscopy in response to various stimuli. Concentration-dependent inhibition of microglial MHC-II expression by pretreatment with LPS (D) and $A\beta_{(1-40)}$ (E). Note, LPS (1 ng) and $A\beta_{(1-40)}$ (50 μM) completely inhibited the INF-γ-induced microglial expression of MHC-II, whereas IL-4 partially reversed the inhibitory effect of LPS and $A\beta_{(1-40)}$ (F). Values of MHC-II intensity are calculated and presented as in Fig. 10.

**Fig. 12A-12C** show that the Neuroprotective effect in hippocampal slices of microglia pretreated with IL-4 in the presence of LPS correlates with IGF-1 expression by microglia. (A) Neural cell death in OHSCs incubated for 24 h with $MG_{(-)}$, $MG_{IFN-\gamma}$ (24 h; 20 ng/ml), $MG_{IL-4}$ (24 h; 10 ng/ml), $MG_{LPS}$ (22 h; 100 ng/ml), or microglia pretreated with the same concentrations of IFN-γ or IL-4 for 2 h prior to addition of LPS for a further 22 h (total incubation time 24 h; $MG_{IFN-\gamma\_LPS}$ or $MG_{IL-4\_LPS}$, respectively). Pretreatment with IL-4 for 2 h conferred significant protection against subsequent exposure to LPS for 22 h. $MG_{IFN-\gamma\_LPS}$ were slightly less cytotoxic than $MG_{(LPS)}$, but the difference was not significant. (B) Identical microglial phenotypes were analyzed for TNF-α and IGF-I mRNA by semi-quantitative RT–PCR. Results of one of two independent experiments are shown. Values represent the relative amounts of amplified mRNA normalized against β-actin in each sample, and are represented as fold of induction relative to $MG_{(-)}$ (means ± SD). (C) Confocal photomicrographs of identical microglia fixated and triple-stained for I-B4 (fluorescein, green), IGF-I (rhodamine, red) and MHC-II (Cy5, blue). Statistical analysis of IGF-I expression demonstrates fluorescence intensity per cell, calculated as a percentage of the intensity measured in $MG_{(-)}$ (defined as 100%; means ± SD; obtained in two independent experiments, each repeated four times). Note, relative to $MG_{(-)}$, the $MG_{IL-4}$ showed a significant

increase in IGF-I expression, which persisted in microglia that were treated for 2 h with $MG_{IL\text{-}4\_LPS}$.

**Fig. 13** shows that IL-4 partially reverses the down-regulation of IGF-I transcripts and the decrease in TNF-α transcripts in $A\beta_{(1-40)}$-primed microglia. Microglia were stimulated with the indicated sequential treatments of IL-4 (10 ng/ml) and $A\beta_{(1-40)}$ (50 μM), each for 12 h. Total RNA was isolated and analyzed for TNF-α and IGF-I mRNA by semi-quantitative RT–PCR. Values represent the relative amounts of amplified mRNA normalized against β-actin in the same sample, and are expressed as fold of induction relative to $MG_{(-)}$ (means ± SD). Results of one of two independent experiments are shown.

**Fig. 14** shows that neutralizing anti-IGF-I antibodies partially inhibit the neuroprotective effect of microglia treated with IL-4 in hippocampal slices. OHSCs were incubated for 24 h with culture medium alone (control) or were co-incubated with $MG_{(-)}$ or with $MG_{IL\text{-}4}$ for 24 h before being added to OHSCs. Neutralizing anti-IGF-I antibodies (ãIGF-I) were added to slices in combination with microglia, as indicated. Statistical analysis shows PI staining intensity, calculated as a percentage of the intensity measured in untreated OHSCs (means ± SD; two-tailed Student's *t*-test; $n = 10$ slices per group). Slices co-incubated with $MG_{(-)}$ and ãIGF-I show a slight increase in neural cell death relative to $MG_{(-)}$-treated slices. The neuroprotective effect of $MG_{(IL\text{-}4)}$ was suppressed by concomitant incubation of the microglia with ãIGF-I.

## DETAILED DESCRIPTION OF THE INVENTION

Neurodegenerative diseases differ in etiology but are propagated similarly. It is shown herein that neuronal loss caused by intraocular injection of aggregated β-amyloid was significantly greater in immunodeficient mice than in normal mice. The neurodegeneration was attenuated or augmented by elimination or addition, respectively, of naturally occurring CD4+CD25+ regulatory T cells (Treg). Vaccination with retina-derived antigens or with the synthetic copolymer glatiramer

acetate (Copolymer-1; Cop-1), but not with β-amyloid, reduced the ocular neuronal loss. In mouse hippocampal slices, microglia encountering activated T cells overcame the cytotoxicity of aggregated β-amyloid. These findings support the concept of "protective autoimmunity", show that a given T cell-based vaccination is protective at a particular site irrespective of toxicity type, and suggest that locally activated T cells induce a microglial phenotype that helps neurons withstand the insult. Alzheimer's and other neurodegenerative diseases might be arrested or retarded by vaccination with Cop-1 or related compounds.

The composition of the invention preferably comprises Copolymer 1, most preferably in the form of its acetate salt known under the generic name glatiramer acetate. Glatiramer acetate has been approved in several countries for the treatment of multiple sclerosis (MS) under the trade name, Copaxone® (a trademark of Teva Pharmaceuticals Ltd., Petach Tikva, Israel). In a most preferred embodiment, the composition of the invention is Copaxone®, used for daily administration for treatment of multiple sclerosis, only that for the purpose of the present invention the composition is administered according to a different regimen, as disclosed herein. This composition is administered in a regimen that confers protective autoimmunity and is sometimes referred to herein as a vaccine for neuroprotective vaccination. However, if desired, the vaccine may contain Copolymer 1 emulsified in an adjuvant suitable for human clinical use.

As used herein, the terms "Cop 1" and "Copolymer 1" are used interchangeably. For the purpose of the present invention, "Cop 1 or a Cop 1-related peptide or polypeptide" is intended to include any peptide or polypeptide, including a random copolymer, that cross-reacts ~~functio~~nally with myelin basic protein (MBP) and is able to compete with MBP on the MHC class II in the antigen presentation, and includes the term glatiramer acetate, the active ingredient of Copaxone®.

The composition or vaccine of the invention may comprise as active agent a random copolymer comprising a suitable quantity of a positively charged amino acid such as lysine or arginine, in combination with a negatively charged amino

acid (preferably in a lesser quantity) such as glutamic acid or aspartic acid, optionally in combination with a non-charged neutral amino acid such as alanine or glycine, serving as a filler, and optionally with an amino acid adapted to confer on the copolymer immunogenic properties, such as an aromatic amino acid like tyrosine or tryptophan. Such compositions may include any of those copolymers disclosed in WO 00/05250, the entire contents of which being hereby incorporated herein by reference.

A preferred copolymer according to this embodiment comprises in combination alanine, glutamic acid, lysine, and tyrosine, of net overall positive electrical charge and of a molecular weight of about 2,000 - 40,000 Da, preferably of about 2,000 -13,000 Da, and is most preferably Copolymer 1 of average molecular weight of about 4,700 - 13,000 Da, but also higher molecular weight forms of Copolymer 1 are encompassed by the present invention. Preferred molecular weight ranges and processes for making a preferred form of Cop 1 are described in U.S. Patent No. 5,800,808, the entire contents of which being hereby incorporated in the entirety. It is clear that this is given by way of example only, and that the vaccine can be varied both with respect to the constituents and relative proportions of the constituents if the above general criteria are adhered to. Thus, the copolymer may be a polypeptide from about 15 to about 100, preferably from about 40 to about 80, amino acids in length, and is preferably the copolymer having the generic name glatiramer acetate.

The Cop 1-related peptide or polypeptide may be any of those disclosed in the above-mentioned US Provisional Application No. 60/518,627 of the present applicants, filed November 12, 2003, hereby incorporated by reference in its entirety as if fully disclosed herein.

In another embodiment, the treatment comprises administering T cells which have been activated in the presence of Cop 1 or a Cop 1-related peptide or polypeptide. Such T cells are preferably autologous, most preferably of the CD4 and/or CD8 phenotypes, but they may also be allogeneic T cells from related donors, e.g., siblings, parents, children, or HLA-matched or partially matched,

semi-allogeneic or fully allogeneic donors. T cells for this purpose are described in USSN 09/756,301 and USSN 09/765,644, corresponding to WO 01/93893, each and all of them hereby incorporated by reference in its entirety as if fully disclosed herein.

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically acceptable carriers or excipients. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the composition and not deleterious to the recipient thereof.

Methods of administration include, but are not limited to, parenteral, e.g., intravenous, intraperitoneal, intramuscular, subcutaneous, mucosal (e.g., oral, intranasal, buccal, vaginal, rectal, intraocular), intrathecal, topical and intradermal routes. Administration can be systemic or local.

The invention will now be illustrated by the following non-limiting examples.

## EXAMPLES

### SECTION I: VACCINATION WITH AUTOANTIGEN OR COP 1 PROTECTS AGAINST β-AMYLOID AND GLUTAMATE TOXICITY

**Materials and methods - Section I**

***(i) Animals.*** Mice were handled according to the Association for Research in Vision and Ophthalmology (ARVO) resolution on the use of animals in research. Male C57Bl/6J wild type, BALB/c/OLA wild type, and nude mice, all specific pathogen-free and aged between 8 and 13 weeks, were supplied by the Animal Breeding Center of The Weizmann Institute of Science (Rehovot, Israel) under germ-free conditions. The mice were housed in a light- and temperature-controlled room and matched for age in each experiment. Mice were anesthetized by i.p. administration of ketamine (80 mg/kg; Ketaset, Fort Dodge, IA) and xylazine (16

mg/kg; Vitamed, Ramat-Gan, Israel). Prior to tissue excision the mice were killed with a lethal dose of pentobarbitone (170 mg/kg; C.T.S., Kiryat Malachi, Israel).

**(ii)** ***Antigens.*** Bovine interphotoreceptor retinoid-binding protein (IRBP) was purified from retinal extracts by affinity chromatography on ConA as described [74]. Bovine S-antigen (arrestin) was prepared from the ConA column flowthrough by the method of Buczylko and Palczewski [75] as modified by Puig et al. [76]. Whole retinal homogenate (WRH) was prepared from syngeneic retinas homogenized in PBS. OVA, Con A, and β-amyloid peptide 1–40 ($A\beta_{1-40}$) were purchased from Sigma (St. Louis, MO). Glatiramer acetate (Copaxone®; Cop-1) was purchased from Teva Pharmaceuticals Ltd. (Petah Tikva, Israel).

**(iii)** ***Immunization.*** Adult mice were immunized with IRBP (50 μg), S-antigen (50 μg), $A\beta_{1-40}$ (50 μg), WRH (600 μg), or Cop-1 (75 μg), each emulsified in an equal volume of CFA (Difco, Detroit, MI) containing *Mycobacterium tuberculosis* (5 mg/ml; Difco). The emulsion (total volume 0.15 ml) was injected s.c. at one site in the flank. Control mice were injected with PBS in CFA or with PBS only.

***(iv) Labeling of retinal ganglion cells.*** Mice were anesthetized as described above and placed in a stereotactic device. The skull was exposed and the bregma was identified and marked. The neurotracer dye FluoroGold (5% solution in saline, Fluorochrome, Denver) was stereotactically injected with a Hamilton syringe, and the skin over the wound was sutured [21].

***(v) Induction of toxicity by injection of glutamate or aggregated $A\beta_{1-40}$.*** The right eyes of anesthetized C57Bl/6J or BALB/c/OLA mice were punctured with a 27-gauge needle in the upper part of the sciera and a Hamilton syringe with a 30-gauge needle was inserted as far as the vitreal body. Each mouse was injected with a total volume of 1 μl of PBS containing L-glutamate (400 nmol; Sigma-Aldrich) or aggregated $A\beta_{1-40}$ (50 μM; Sigma-Aldrich).

***(vi) Assessment of retinal ganglion cell survival.*** At the end of the experimental period the mice were given a lethal dose of pentobarbitone (170 mg/kg). Their eyes were enucleated and the retinas were detached, prepared as

flattened whole mounts in 4% paraformaldehyde in PBS, and labeled cells from four to six fields of identical size (0.076 $mm^2$) were counted [9, 21]. The average number of RGCs per field was calculated for each retina. The number of RGCs in the contralateral (uninjured) eye was also counted, and served as an internal control.

***(vii) In-situ detection of cell death by terminal deoxynucleotidyl transferase DNA (TUNEL).*** Mice were killed 48 h after intraocular glutamate injection and their eyes were removed and processed for cryosectioning. Frozen sections were fixed in 3.7% formalin for 10 min at room temperature and washed twice with PBS. The sections were transferred to 100% methanol for 15 min at –20°C, washed twice for 5 min in ethanol 100%, 95% and 70% successively, and then incubated for 10 min with PBS. For permeabilization, proteases were digested with proteinase K for 20 min at room temperature. The open ends of the DNA fragments were labeled using an in-situ apoptosis detection kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The labeled ends were detected using the fluorescein detection kit supplied with a streptavidin-fluorescein conjugate. The fluorescein-stained cells were visualized using a fluorescence microscope.

***(viii) Preparation of splenocytes depleted of CD4+CD25+ regulatory T cells.*** Splenocytes prepared by a conventional procedure were incubated with rat anti-mouse PE-conjugated CD25 antibody, and this was followed by incubation with anti-PE beads (Becton-Dickinson, Bactlab Diagnostic, Haifa, Israel). After being washed, the splenocytes were subjected to AutoMacs (Miltenyi Biotec, Bergisch Gladbach, Germany) with the "deplete sensitive" program. Recovered populations were analyzed by FACSsort (Becton Dickinson, Franklin Lakes, NJ) [25].

***(ix) Preparation of activated naïve T cells.*** Lymph nodes (axillary, inguinal, superficial cervical, mandibular, and mesenteric) and spleens were harvested and mashed. T cells were purified (enriched by negative selection) on T cell columns (R&D Systems). The enriched T cells were incubated with anti-CD8 microbeads (Miltenyi Biotec), and negatively selected CD4+ T cells were incubated with PE-conjugated anti-CD25 antibodies (30 µg/$10^8$ cells) in PBS/2% fetal calf serum.

They were then washed and incubated with anti-PE microbeads (Miltenyi Biotec) and subjected to magnetic separation with AutoMACS. The retained cells were eluted from the column as purified CD4+CD25+ cells (Treg). The negative fraction (effector T cells, Teff), consisting of CD4+CD25– T cells, was further activated for 4 days, in medium containing $5\times10^5$ cells/ml, with spleen-derived APC (irradiated with 3000 rad), and 0.5 μg/ml anti-CD3 antibodies, supplemented with 100 units of mouse recombinant IL-2 (mrIL-2; R&D Systems).

***(x) Preparation of antigen-specific activated lymphocytes from immunized mice.*** Ten days after immunization, the mice were killed and their draining lymph nodes were excised and pressed through a fine wire mesh. The washed lymphocytes ($2\times10^6$ cells/ml) were activated with the relevant antigens ($IRBP_{1-20}$ or aggregated $A\beta_{1-40}$, each at 10 μg/ml) in stimulation medium containing RPMI supplemented with L-glutamine (2 mM), 2-mercaptoethanol ($5\times10^{-5}$ M), penicillin (100 IU/ml), streptomycin (100 IU/ml), and autologous mouse serum 1% (vol/vol). After incubation for 48 h at 37°C, 90% relative humidity, and 7% $CO_2$ the lymphocytes were washed with PBS, counted, and injected intraperitoneally into autologous mice not more than 1 h after intravitreal injection of a toxic dose (50 μM) of aggregated $A\beta_{1-40}$.

***(xi) Microglial cultures.*** Microglia were purified from the cerebral cortices of newborn (day 0) BALB/c/OLA mice, as described [53]. IFN-γ (20 ng/ml; R&D Systems), β-amyloid (Sigma-Aldrich; aggregated $A\beta_{1-40}$ 25 μM), or activated T cells ($1.5\times10^5$ per well) were added to the culture medium for 12 h. After treatment, microglia were washed three times with PBS and prepared for application on hippocampal slices.

***(xii) In-vitro model of hippocampal slices.*** BALB/c/OLA mice, aged 8–10 days, were decapitated and their brains were rapidly removed under sterile conditions and placed in ice-cold preparation medium consisting of minimum essential medium (MEM; Gibco, Carlsbad, CA) with 1% L-glutamine (Gibco) at pH 7.35. The frontal pole was removed and the brains were cut into 350-μm

horizontal slices on a vibratome (Pelco, Redding, Germany), beginning at the ventral surface. Slices containing the hippocampi were cultured on Falcon cell culture inserts, pore size 0.4 μm (Becton Dickinson), in 6-well plates. The cultivation medium contained 50% MEM, 25% Hanks balanced salt solution (Gibco), 25% normal horse serum, 2% glutamine, 10 μg/ml insulin–transferrin–sodium selenite supplement (Boehringer Mannheim, Mannheim, Germany), 2.64 mg/ml glucose (Braun, Melsungen, Germany), 0.1 mg/ml streptomycin, 100 U/ml penicillin, and 0.8 μg/ml vitamin C (all from Sigma-Aldrich). The organotypic hippocampal slice cultures (OHSCs) were incubated at 35°C in a humidified atmosphere with 5% $CO_2$ for 24 h, during which time the slices were either left untreated or treated with $4\times10^5$ microglia per well. Tissue loss was assessed by addition of propidium iodide (PI) (5 μg/ml; Sigma) to the medium for 30 min at the end of the incubation period. Excess PI was then washed away with cultivation medium, and the slices were prepared for microscopy and visualized. To quantify neural cell death in the OHSCs, PI intensity in each slice was assessed by use of Image-Pro software (Media Cybernetics, Carlsbad, CA). PI staining intensity for a specific treatment was compared to that of the untreated control, using a two-tailed Student's *t*-test.

**EXAMPLE 1. Retinal proteins can evoke a protective T cell-based response to glutamate intoxication.**

We have shown previously that mice of different genetic backgrounds differ in their ability to resist injurious conditions [9, 16, 17]. The differences were attributed, at least in part, to strain-related variations in the ability to manifest a T cell-dependent protective response [16]. In view of the observed failure of myelin proteins to protect mice against glutamate toxicity in the eye and the successful protection against glutamate toxicity in rats by retinal proteins [9, 11], we were interested in examining whether immunization of mice with retinal proteins would improve their neuronal survival after exposure to glutamate toxicity, and if so, whether the same vaccination would be effective against other threatening

compounds (such as aggregated β-amyloid) injected into the same site. Glutamate (400 nmol) was injected into the right eyes of C57Bl/6J mice, and 48 h later we examined retinal cryosections subjected to terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL). Apoptotic cell death was observed in the RGC layer (Fig. 1A). When mice of this strain were vaccinated with a homogenate of whole retinal proteins (WRH) in CFA 6 days before being injected with a toxic dose of intraocular glutamate (400 nmol), examination 1 week later disclosed survival of significantly more RGCs than that seen in age- and strain-matched control mice injected with PBS emulsified in CFA (2239.7 ± 153.3 and 1480.6 ± 176.2, respectively, $P < 0.0001$; two-tailed Student's *t*-test). This finding indicated that vaccination with the retinal components had increased the ability of the immunized mice to withstand glutamate toxicity. Fig. 1B shows that the vaccination significantly reduced RGC loss, expressed as a percentage of the numbers in normal controls (mean ± SEM; Fig. 1B).

In light of the above results, and to further test our hypothesis that the evoked protection against glutamate toxicity is an outcome of a T cell-mediated response to the retinal self-antigens, we examined whether immunization with the specific eye-resident antigens interphotoreceptor retinoid-binding protein (IRBP) or S-antigen (retinal arsenin), rather than with the retinal homogenate, can protect RGCs against glutamate toxicity. Immunization of C57Bl/6J mice, 10 days before glutamate injection, with IRBP emulsified in CFA resulted in survival of significantly more RGCs than in glutamate-injected C57Bl/6J mice immunized with PBS/CFA (1996 ± 49.53 and 1649 ± 43, respectively, $P < 0.0008$; two-tailed Student's *t*-test; Fig. 1C). Immunization with the retinal self-antigen S-antigen in CFA resulted in a similar increase in neuronal survival relative to immunization with PBS/CFA (2160 ± 38 and 1648 ± 37, respectively, $P < 0.0001$; two-tailed Student's *t*-test; Fig. 1D). For ease of comparison, the results in the figure are presented as the loss of neurons expressed as a percentage of the number of RGCs in strain-matched normal retinas.

It should be emphasized that the retinal self-proteins IRBP and S-antigen, both of which are capable of causing uveitis in susceptible mice [18, 19] but were

used here for purposes of protection, are not intended for development as a therapeutic vaccination; this is purely an experimental paradigm, used here for proof of concept, that supports our previous contention that the same T cells can be both protective and destructive, and that their actual effect is a reflection of the tissue context, the quantity of T cells, and the timing of their activities in the tissue [11, 20, 21].

**EXAMPLE 2. Ability to withstand the toxicity of β-amyloid is T cell-dependent.**

Having shown that the physiologically relevant antigen for protection against neurotoxicity is not the toxic compound itself (glutamate) but a self-antigen that resides in the site of damage, we then examined whether the same vaccination might be beneficial against different toxic self-compounds provided that the toxicity is restricted to the same site. We tested this hypothesis by examining the effect of the vaccination on the toxicity of aggregated β-amyloid. Aggregated β-amyloid $(A\beta)_{1-40}$ (5 or 50 μM) was injected into the right eyes of C57Bl/6J mice. This model (intraocular injection of β-amyloid) was chosen not because of the supposed association of this compound with the optic neuropathy in Alzheimer's disease [13, 22] but because β-amyloid is capable of causing RGC death [23], and therefore its use allows us to further explore the concept of antigenic specificity. Surviving RGCs were counted 1 or 2 weeks after ocular injection of aggregated β-amyloid. After 1 week the numbers of viable RGCs were 2257 ± 77 (5 μM injection) and 2071 ± 30 (50 μM injection), and after 2 weeks they were 2062 ± 41 (5 μM) and 1952 ± 21 (50 μM). A total of 3445 ± 57 neurons were counted in naïve mice. Under the same experimental conditions, toxicity caused by injection of the vehicle alone did not affect more than 5% of RGCs in the normal retina. Fig. 2A shows the β-amyloid-induced neuronal loss expressed as a percentage of the average number of RGCs in normal wild-type retina. Figs. 2B and C show representative

photomicrographs of whole-mounted retinas excised from mice after intraocular injection of PBS and aggregated $A\beta_{1-40}$, respectively.

To determine whether the ability of naïve mice to withstand the toxicity of aggregated $A\beta_{1-40}$ is T cell-dependent, we compared RGC survival in wild type and nude (nu/nu) BALB/c/OLA mice 2 weeks after intraocular injection of aggregated $A\beta_{1-40}$ (50 μM). Significantly more neurons survived in the injected wild-type mice (2316 ± 53) than in their T cell-deficient counterparts (1779 ± 147; $P < 0.01$). The choice of BALB/c/OLA mice for this experiment was based on a previous observation that the T cell-dependent ability of this strain to withstand the consequences of CNS injury is significantly better than that of C57Bl/6J mice [9, 16, 24], and thus any differences resulting from the absence of T cells would be more easily detectable in the BALB/c/OLA mice. Fig. 2D shows neuronal loss as a percentage of the number of neurons in normal retinas. Figs. 2E and 2F show representative micrographs of retinas from wild-type and nude mice, respectively, after intraocular injection of aggregated $A\beta_{1-40}$. These results support the contention that the strain is a factor in the ability of neural tissue to withstand ocular toxicity, and show that strain-related differences in that respect are related not to the type of insult but to the ability to harness a well-controlled T cell-dependent immune response.

To further test our working hypothesis that the T cell specificity needed for neuroprotection is directed not against the threatening compound but against self-antigens that reside in the site of the lesion, we subjected C57Bl/6J mice to intraocular toxicity of aggregated $A\beta_{1-40}$ and then immunized them with the IRBP-derived peptide (which, as in the case of glutamate toxicity (Fig. 1C), is protective against the intraocular toxicity of aggregated $A\beta_{1-40}$). After vaccination, the loss of RGCs induced by aggregated $A\beta_{1-40}$ was significantly smaller than that observed in mice immunized with PBS/CFA (RGC survival was 2307 ± 62 for IRBP/CFA and 1840 ± 56 for PBS/CFA; Fig. 3A). We also immunized C57Bl/6J mice with the non-aggregated (non-toxic) form of the β-amyloid peptide before injecting them

intraocularly with $A\beta_{1-40}$. After vaccination with β-amyloid/CFA, the loss of RGCs induced by aggregated $A\beta_{1-40}$ did not differ significantly from that observed in mice immunized with PBS/CFA (RGC survival was 1743 ± 55 and 1831 ± 45, respectively; Fig. 3B).

To verify that the observed vaccination-induced protection is T cell-dependent, we prepared primary T cells directed against IRBP and S-antigen or against IRBP only. After their activation ex vivo, the lymphocytes ($1.2\times10^7$ cells) were transferred into naïve C57Bl/6J mice freshly exposed to toxicity of glutamate or aggregated $A\beta_{1-40}$. Significantly more RGCs survived in mice that received lymphocytes activated with IRBP + S-antigen than in mice immunized with lymphocytes activated by the non-CNS antigen OVA (2220 ± 38 compared to1652 ± 56, $P < 0.001$; two-tailed Student's *t*-test; Fig 4A). RGC survival in mice that received OVA-activated T cells did not differ significantly from that in naïve mice injected with glutamate (1652.6 ± 56 and 1535.6 ± 74, respectively; not shown). Results are expressed as the percentage increase in neuronal survival relative to survival in control mice (Fig. 4A).

Similarly, T cells specific to IRBP or to non-aggregated $A\beta_{1-40}$ obtained from immunized mice were prepared and activated ex vivo, and then passively transferred into C57Bl/6J mice exposed to toxicity of aggregated $A\beta_{1-40}$. Passive transfer of the T cells activated with IRBP-derived peptide was beneficial in mice that were injected intraocularly with aggregated $A\beta_{1-40}$, as shown by the significantly smaller loss of RGCs in these mice than in normal control mice (Fig. 4B). In contrast, in mice that received a passive transfer of T cells specific to non-aggregated $A\beta_{1-40}$, the loss of RGCs after intraocular injection with a toxic dose of aggregated $A\beta_{1-40}$ differed only slightly from that in mice treated with PBS.

These findings confirmed that the protection achieved by active vaccination with IRBP (Fig. 3A) against the toxicity of aggregated $A\beta_{1-40}$ was T cell-mediated. The failure of T cells directed to the aggregated $A\beta_{1-40}$ itself to confer protection is in line with observations in our laboratory that microglia, upon encountering

aggregated $A\beta_{1-40}$, fail to express MHC class II (MHC-II). Consequently, such microglia fail to present β-amyloid to the T cells, with the result that even if β-amyloid-specific T cells home to the CNS they will not be locally activated (Butovsky et al., unpublished observations). To fight β-amyloid toxicity, a more appropriate choice would therefore be antigens that reside in the site and can be presented to homing T cells.

The results summarized above suggest that an appropriate choice for vaccination in order to fight β-amyloid toxicity would be antigens that reside in the site of degeneration and that can be presented to the homing T cells. Because of the diversity of the human histocompatibility complex, vaccination with self-antigens cannot be assumed to be safe for therapeutic purposes. In searching for a safe vaccine we examined the efficacy of vaccination with the synthetic antigen glatiramer acetate, also known as Copolymer 1 (Cop-1) [9, 15], which was previously shown to act as a partial agonist or an altered peptide ligand in mimicking the effect of a wide-range of self-reactive T cells without causing an autoimmune disease [9]. Significant protection against toxicity of aggregated β-amyloid was obtained by vaccinating C57Bl/6J mice with this copolymer 7 days before they were injected intraocularly with a toxic dose of aggregated $A\beta_{1-40}$ (Fig. 5). RGC survival in Cop-1-treated and PBS-treated mice was 1939 ± 80 and 1617 ± 43, respectively $P < 0.01$; two-tailed Student's *t*-test.

**EXAMPLE 3. Naturally occurring regulatory $CD4^+CD25^+$ T cells restrict the body's ability to withstand β-amyloid toxicity in the retina.**

The above observation that in the absence of intervention the ability of mice to withstand the toxicity of aggregated $A\beta_{1-40}$ is T-cell dependent (Fig. 2) prompted us to investigate whether the ability of the neural tissue to spontaneously withstand the toxicity of aggregated β-amyloid is suppressed by the naturally occurring regulatory $CD4^+CD25+$ T cells (Treg), as described in the case of other CNS insults [25, 26]. If so, elimination or weakening of such control might serve as an

additional way to harness the autoimmune T cells needed for protection against β-amyloid-associated neurodegenerative conditions. We therefore examined whether the ability of the murine neural tissue to withstand the toxicity of aggregated $A\beta_{1-40}$ could be boosted by removal of Treg. In adult C57Bl/6J mice that had undergone thymectomy 3 days after birth (a procedure that results in Treg depletion [27, 28]), significantly more RGCs survived exposure to aggregated $A\beta_{1-40}$ than in matched non-thymectomized controls (2251 ± 53 and 1918 ± 94, respectively; $P < 0.01$; two-tailed Student's *t*-test Fig. 6A). In a complementary experiment, nude mice of the BALB/c/OLA strain were replenished with $4.5\times10^7$ wild-type splenocytes from which the Treg population had been removed ex vivo. As controls, we used nude mice replenished with the same number of splenocytes, which were obtained from whole spleens of wild-type mice and therefore contained both Treg and effector T cells (Teff). Three days after replenishment, the recipient mice were injected with a toxic dose of aggregated $A\beta_{1-40}$, and surviving RGCs were counted 2 weeks later. Significantly fewer RGCs died in the mice replenished with splenocytes depleted of Treg than in mice replenished with a normal splenocyte population; in both groups, however, fewer RGCs died than in the group of untreated nu/nu mice injected with aggregated $A\beta_{1-40}$ (RGC survival was 2412 ± 61, 2246 ± 101, and 2080 ± 56, respectively; Fig. 6B). These findings suggest that Treg normally down-regulate the ability of the neural tissue to spontaneously withstand aggregated $A\beta_{1-40}$ toxicity. PCR testing for Foxp3 expression, found to be associated with Treg [29], confirmed that the Treg were Foxp3-positive whereas $CD4^+CD25^-$ T cells were Foxp3-negative (Fig. 6C).

**EXAMPLE 4. T cells prevent microglia from developing an inflammatory cytotoxic phenotype.**

It was previously proposed by the inventors that one way in which the autoimmune T cells help to fight off destructive self-compounds is by controlling the activity of microglia [12]. Using organotypic hippocampal slice cultures

(OHSCs), our group showed that after rat microglia are pretreated with aggregated $A\beta_{1-40}$ they become cytotoxic to neural tissue and their ability to express MHC-II is suppressed (Butovsky et al., unpublished observations). We therefore carried out an in-vitro experiment to determine whether murine microglia exposed to aggregated $A\beta_{1-40}$ also become cytotoxic, and if so, whether activated T cells can overcome the toxicity. After exposure of mouse microglia to aggregated $A\beta_{1-40}$, their addition to mouse OHSCs resulted in significantly more neuronal death than that seen in OHSCs that were untreated or were treated with naïve microglia (Fig. 7). The loss was significantly reduced, however, if the added microglia, at the time of their exposure to aggregated $A\beta_{1-40}$, had also been exposed to activated effector ($CD4^+CD25^-$) T cells (Fig. 7A). Representative micrographs of variously treated OHSCs and untreated controls are shown in Fig. 7B (1–4). These findings support the contention that exposure of microglia to activated T cells in suitably controlled amounts not only prevents the microglia from becoming cytotoxic, but also enables them to become neuroprotective. It should be noted that the microglial toxicity assayed in vitro does not reflect the lack of MHC-II expression, as this bioassay does not require antigen presentation.

**Discussion**

The results of the present invention as described in Section I above suggest that in developing a therapeutic vaccination to counteract the toxicity caused by accumulation of aggregated $A\beta_{1-40}$ and other toxic agents such as glutamate, the same vaccine can be used provided that the toxic agents are all located—as is often the case—in the same site. In the mouse model used in the present invention, two neurotoxic self-compounds were injected into the eye, and protection against both of them was achieved by vaccination with the same antigens, namely peptides derived from proteins that reside in the eye. We interpret this finding as proof of principle that dominant self-antigens constitutively residing in a site of damage are the self-protective antigens against threatening conditions at this site. We further

show that depletion of the naturally occurring CD4+CD25+ regulatory T cells (Treg) can increase the spontaneous response to such antigens and thus the ability to withstand the toxic effect of aggregated β–amyloid. As a therapeutic strategy, however, we propose vaccinating with Copolymer 1, a synthetic weak agonist of self-antigens [9, 15, 30, 31], rather than with the site-specific self-proteins themselves, because the former can be used as a protective vaccine without risk of inducing an autoimmune disease, a potential hazard associated with inherently inadequate control of autoimmunity [32-35]. As an alternative strategy, we propose the use of any manipulation that will weaken the activity of Treg [36].

The amino acid glutamate is a major neurotransmitter in the CNS [1, 37]. When the optimal physiological concentration of glutamate is exceeded, for example as a result of synaptic activity, it is restored to normal by local mechanisms [38-40]. These include transporters that help to remove excessive glutamate, as well as enzymes that convert it to glutamine [41-43]. Glutamate is normally buffered via its active transport from the extracellular milieu, an activity carried out mainly by astrocytes [40] but also by neurons and microglia [44]. When the amount of glutamate exceeds the buffering capacity of the neural cells, it becomes neurotoxic [38].

Neurodegenerative disorders such as Parkinson's, Alzheimer's, prion, motor neuron diseases, and other devastating chronic neurodegenerative syndromes have several features in common, including the accumulation of self-proteins that have either become aggregated or undergone conformational changes [45]. In the case of Alzheimer's disease, accumulation of aggregated $A\beta_{1-40}$ is potentially a major cause of neuronal toxicity [2]. The present results support the contention that the β-amyloid peptide in its aggregated form (found in senile plaques) [46] has a toxic effect in the CNS, not only because it is directly toxic to neurons [23, 47] but also because it apparently induces microglia to adopt a cytotoxic phenotype. In addition, the failure of β-amyloid vaccination to protect against β-amyloid-induced stress in the eye is in line with observations from our laboratory that cell-surface MHC-II

expression is impaired in microglia encountering aggregated β-amyloid (Butovsky et al., unpublished observations).

In the past, it was generally assumed that because activated microglia are seen in the context of neurodegenerative diseases, these cells contribute to the ongoing degeneration [48, 49]. Accordingly, a substantial research effort was devoted to achieving their suppression.

The present invention indicates that an alternative approach to the problem necessitates modulation of the microglial phenotype, thereby not only minimizing the risk carried by malfunctioning microglia but also exploiting microglial assistance in withstanding the destructive effects of aggregated $A\beta_{1-40}$ and other toxic agents associated with ongoing degeneration such as glutamate and oxidative stress. The phenotype acquired by microglia exposed to activated T cells is not destructive insofar as it does not produce inflammation-associated enzymes or promote redox imbalance [12]. Thus, T cells that can be locally activated, irrespective of the identity of the antigen(s) residing in the damaged site, can transform the adjacent microglial population from an enemy into a friend.

Memory T cells, unlike naïve T cells, home to the lesion site [50-52], and those that encounter their specific APC become activated there. It is possible that T cells directed to self-antigens residing in the lesion site (other than those directed to β-amyloid) can overcome the negative effect on microglia caused by the aggregated $A\beta_{1-40}$ residing in the same site. Thus, not only are the activated T cell-dependent microglia not toxic, but they can also hold a dialog with T cells [53] resulting in production of cytokines (such as IFN-γ and IL-4) and growth factors [7, 14, 31, 54], which can further activate the local microglia to clear away potentially harmful cell debris and toxic compounds [12].

It is important to note that OHSC assay cannot be used to monitor the activity of microglia as APC, since T cells do not participate in this bioassay. In independent studies, however, it was shown by our group that expression of MHC-II in microglia correlates with the observed neuroprotection [53]. It was further shown that protection by microglia is correlated with MHC-II but does not depend

on it, and that factors produced by cytotoxic microglia (TNFα, COX-2, NO, and others) interfere with MHC-II expression (Butovsky et al., unpublished observations). Moreover, microglia from invariant chain knockout mice can be protective [55].

In the present invention, we observed strain-related differences in the ability of mice to withstand the toxicity of aggregated $A\beta_{1-40}$. This observation is reminiscent of previous findings, where mice were shown to be capable of withstanding the consequences of axonal injury and glutamate toxicity [16, 17, 56, 57]. The present results are also in line with our contention that naturally occurring CD4+CD25+ regulatory T cells constitutively control the ability to withstand neurodegenerative conditions. Although these cells are key participants in protection against autoimmune disease [58], they limit the ability to fight degeneration [25]. We have previously postulated that the presence of these cells reflects an evolutionary compromise between the need for autoimmune protection and the risk of developing an autoimmune disease because of inadequate control of the immune response [25, 59], the latter being an outcome of the failure of Treg to display optimal suppressive activity [58]. In rats or mice devoid of Treg, the susceptibility to autoimmune disease development is increased, despite the benefit in terms of protection against injurious conditions Therefore, one of the aims of neuroprotective therapy is to weaken Treg. The prospect of weakening these cells in a well-controlled way has now become feasible, following the discovery of a brain-derived compound that can selectively affect the trafficking of Treg to sites of damage and attenuate their suppressive activity [36]. Thus, pharmacological intervention with a compound that mimics the physiological weakening (but not blocking) of Treg might provide a way to boost the T cell-based self-defense. The fact that a strain that is susceptible to EAE is also limited in its ability to derive the benefit of a spontaneous T cell-dependent autoimmune response, coupled with the suppressive effect of Treg on spontaneous autoimmunity, might suggest that Treg is malfunctioning in this strain [60] or that the ratio between Treg and the population

of effector $CD4^+$ $CD25^-$ T cells directed against self-antigens in this strain is not optimal, as suggested by preliminary results in our laboratory [60-62].

It was shown by our group that the same autoimmune T cells can be both supportive and destructive [63]. Accordingly, in animals that are inherently susceptible to autoimmune disease the protocol used for eliciting the T cell response critically affects the outcome. Thus, a strong adjuvant might lead to an autoimmune response whose benefit is offset by its persistence or intensity [20, 64]. In such susceptible strains, however, autoimmune response to CNS might not be expressed early enough to be accommodated within the therapeutic window, or it might fail to meet other requirements, such as timely shut-off [65]. Moreover, in susceptible strains devoid of immune cells (SCID) and thus lacking a T cell-based regulatory mechanism, passive transfer of encephalitogenic T cells causes EAE [63], but is not sufficient for conferring any neuroprotection [63] and possibly even having a destructive effect on the neurons [66]. In contrast, when $CD4^+CD25^+$ regulatory T cells are passively transferred into SCID mice [60], they can have a protective effect similar to that of encephalitogenic T cells passively transferred into the wild type [6, 7, 67]. In animals that are inherently resistant to autoimmune diseases the likelihood that the spontaneously evoked response to a CNS injury will be destructive is very low; on the other hand, it might be too weak to be beneficial and need boosting. Thus, whether or not autoimmunity will be beneficial under severe conditions in susceptible strain is determined by both regulation and context. For therapies capable of meeting the criteria of both resistant and susceptible strains without running the risk of negative side effects, the use of weak synthetic antigens such as Cop-1 or other related compounds deserves consideration. Such a strategy, unlike vaccination with a peptide derived from a toxic antigen such as β-amyloid, can potentially provide risk-free benefit. Moreover, the same safe antigen can be used for protection at different sites of degeneration, a situation that is often required in patients.

The results herein further support the contention that the way in which the body harnesses the immune system for protection against neurodegenerative

conditions is via a T cell-dependent pathway. In addition, they strengthen the notion that in adopting a therapeutic approach to neurodegenerative diseases characterized by protein deposition, the antigen selected for vaccination should not be the disease-specific protein (such as aggregated $A\beta_{1-40}$ in Alzheimer's disease, Lewy bodies in Parkinson's disease, or prion protein in prion disease) [68, 69], but a peptide derived from an immunodominant self-protein that resides at the site of CNS damage. The relevant peptide can also be a cryptic self-peptide, an altered self-peptide, or a non-self peptide that cross-reacts weakly with self [7, 9, 20, 70].

The T cell-based vaccination in this study protected mice from the neurodegenerative effects of existing aggregated $A\beta_{1-40}$. The proposed strategy does not argue against the possible benefit of antibodies specific to Aβ-amyloid [68, 71-73] as long as the peptide used for vaccination is not encephalitogenic. The two approaches, rather than being mutually antagonistic, might complement one another.

## SECTION II: AGGREGATED β-AMYLOID, ADAPTIVE IMMUNITY AND MICROGLIA


### Abstract

'Protective autoimmunity' refers to a well-controlled T cell-mediated anti-self response that helps the body resist neurodegeneration. Using an *in-vitro* assay of hippocampal slices to assess the cytotoxic or protective effect of microglia in neural tissue we show that interferon (IFN)-γ and especially interleukin (IL)-4, characteristic Th1 and Th2 cytokines, respectively, endow microglia with a protective phenotype. In contrast, aggregated β-amyloid, like bacterial cell wall-derived lipopolysaccharide (LPS), evoked a cytotoxic microglial response. Cytotoxicity was correlated with a signal-transduction pathway that down-regulated MHC-II expression through the MHC II-transactivator and the invariant chain. Protection by IL-4 was attributed to down-regulation of TNF-α and up-regulation of insulin-like growth factor I (IGF-I). These findings suggest that beneficial or

harmful expression of the local immune response in the damaged CNS depends on how microglia interpret the threat, and that a well-regulated T cell-mediated response enables microglia to alleviate rather than exacerbate stressful situations in the CNS.

**ABBREVIATIONS II: APC**, antigen-presenting cell; **MHC-II**, class II major histocompatibility proteins; **Ii**, invariant chain; **LPS**, lipopolysaccharide; **IFN**, interferon; **PI**, propidium iodide; **TNF**, tumor necrosis factor; $A\beta_{(1-40)}$, β-amyloid (1–40) peptide; **OHSCs**, organotypic hippocampal slice cultures; **CIITA**, MHC class II transactivator; **IGF-I**, insulin-like growth factor I

## Introduction

Degeneration of CNS neurons, whether caused by an acute insult or by a chronic process, is always associated with local inflammation, an immune response that was generally assumed to have a detrimental effect on the tissue. Research findings over the last few years, however, have yielded the concept of protective autoimmunity, in which a well-controlled immune response directed against specific CNS self-antigens is viewed as the body's defense mechanism against self-derived threats, including destructive self-compounds associated with neurodegenerative disorders (Moalem et al., 1999). Accumulating knowledge of the mechanism by which autoimmune T cells facilitate CNS protection and repair has enabled scientists to better understand the etiology of neurodegenerative diseases in general, and in particular what turns a self-compound into an enemy (Itagaki et al., 1989).

It is very likely that T helper (Th) cells orchestrate the wound-healing functions of resident microglia and invading macrophages in the CNS, including removal of myelin debris and other potential threats to the tissue (Schwartz et al., 2003). Recent studies by our group suggest that T cells can regulate the early onset and timely shut-off of an immune response by appropriate activation of the

microglia (Schwartz et al., 2003; Shaked et al., 2004a). According to this view, optimal timing of the immune response, achieved through its rigorous control, has a beneficial effect on the damaged tissue (Kipnis et al., 2002b).. An additional contribution of the T cells is their secretion of neurotrophins, possibly as an outcome of their dialog with resident microglia upon activation of the T cells by their specific antigen-presenting cells (APCs) in the CNS (Hohlfeld et al., 2000).

In apparent contradiction to the scenario suggested by our group, inflammation in neurodegenerative diseases has long been thought to contribute to the pathology (Popovich et al., 1999). Microglia activation appears to be the most prominent inflammatory component in neurodegenerative deceases such as Alzheimer's disease (AD). Neuroinflammation hypothesis states that aggregated β-amyloid protein, presence in senile plaques of AD patients, activates microglial cells to produce neurotoxic substances that cause neurodegenerative changes (McGeer et al., 1987; Akiyama et al., 2000). Microglial cells — the CNS equivalent of macrophages — in their normal status, these cells hold themselves in readiness to perform immune or neural tasks, depending on how they interpret the body's needs (reviewed in (Streit, 2002)). They are capable of sensing homeostatic disturbances and recognize danger signals that can rapidly trigger their transformation from 'resting' condition into an alert or 'activated' state. Once activated, they serve primarily to defend and protect the CNS via mechanisms of innate and adaptive immunity. It is assumed generally that activated microglia kill neurons even though there is hardly any direct evidence to support it, at least not from *in vivo* studies, which often suggest the opposite, namely that microglia provide neuroprotection (Rapalino et al., 1998; Streit, 2002).

We argue, however, that the "protective versus destructive" dichotomy of microglial activity in the context of neurodegeneration does not necessarily reflect contradictory views, but rather supports the concept that microglia function as stand-by resident immune cells (Kerschensteiner et al., 1999; Olsson et al., 2003; Schwartz et al., 2003). If these sentinels fail (as a result of their inappropriate activation) to correctly read the incoming stress signals, they will not develop the

appropriate phenotype needed to fight off the threat. Thus, whether the local immune response will be harmful or beneficial apparently depends on how the microglia interpret the threat (Hausler et al., 2002).

The present study was undertaken in an attempt to examine whether and how adaptive immunity renders the microglial phenotype protective, to determine if and how self-compounds that are manifested in forms associated with neurodegenerative disorders interfere with the ability of microglia to protect the tissue against their harmful effects, and to find ways in which such threats can be circumvented by the adaptive immune system.

**Materials and methods Section II**

***(xiii) Reagents.*** Lipopolysaccharide (LPS; containing less than 1% contaminating protein) from *Escherichia coli* 0127:B8 and β-amyloid [amyloid protein fragment 1–40 ($A\beta_{1-40}$)] were purchased from Sigma-Aldrich, St. Louis, MO. The $A\beta_{(1-40)}$ peptide was dissolved in endotoxin-free water, and β-amyloid aggregates were formed by incubation of $A\beta_{(1-40)}$, as described (Ishii et al., 2000). Recombinant rat interferon (IFN)-γ and interleukin (IL)-4, both containing endotoxin level less than 0.1 EU/μg of cytokine, goat anti-rat tumor necrosis factor (TNF)-α neutralizing antibody (Ab) (ãTNF-α) (endotoxin less than 10 ng/mg of Ab), goat anti-mouse/rat insulin-like growth factor 1 (IGF-I), and neutralizing antibody (ãIGF-I) (endotoxin less than 0.1 EU/μg of Ab) were obtained from R&D Systems (Minneapolis, MN).

***(xiv) T-cell line specific to myelin basic protein.*** A T-cell line specific to myelin basic protein (MBP; $T_{MBP}$) was generated from lymph node cells of Sprague-Dawley (SD) rats immunized with MBP. Cells were propagated and re-stimulated as previously described (Moalem et al., 1999).

***(xv) Isolation of rat microglial cells*** Microglial purity was determined according to previously published protocols (Zielasek et al., 1992). In brief, brains from neonatal SD rats (P0–P2; The Weizmann Institute of Science, Israel) were stripped of their meninges and minced with scissors under a dissecting microscope

(Zeiss, Stemi DV4, Germany) in Leibovitz-15 medium (Biological Industries, Beit Ha-Emek, Israel). After trypsinization (0.5% trypsin, 10 min, 37°C, 5% $CO_2$), the tissue was triturated. The cell suspension was washed in culture medium for glial cells [Dulbecco's modified Eagle's medium (Gibco; Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS; Sigma-Aldrich), L-glutamine (1 mM), sodium pyruvate (1 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml)] and cultured at 37°C/5% $O_2$ in 75-$cm^2$ Falcon Primaria tissue-culture flasks (BD Biosciences, Franklin Lakes, NJ) coated with poly-D-lysine (PDL, 10 μg/ml; Sigma-Aldrich) in borate buffer (2.37 g borax and 1.55 g boric acid dissolved in 500 ml sterile water, pH 8.4) for 1 h, and then rinsed thoroughly with sterile, glass-distilled water. Half of the medium was changed after 6 h in culture and every second day thereafter, starting on day 2, for a total culture time of 7–10 days. Microglia were shaken off the primary mixed brain glial cell cultures (150 rpm, 37°C, 6 h) with maximum yields between days 7 and 10, seeded ($10^5$ cells/ml) into PDL-pretreated 6-well (4 ml/well) or 24-well (1 ml/well) plates (Corning, Corning, NY), and grown in culture medium for microglia [RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% FCS, L-glutamine (1 mM), sodium pyruvate (1 mM), β-mercaptoethanol (50 μM), penicillin (100 U/ml), and streptomycin (100 μg/ml)]. The cells were allowed to adhere to the surface of a PDL-coated culture flask (1 h, 37°C, 5% $CO_2$), and nonadherent cells were rinsed off. Only microglia that were more than 95% pure were used for *in-vitro* cultures and for organotypic hippocampal slice cultures (OHSCs). Prior to use the microglial cultures were incubated with the indicated reagents, antibodies or cells for the indicated times at 37°C.

***(xvi) Organotypic hippocampal slice cultures.*** Brains of decapitated SD rats (8–10 days old) were rapidly removed and placed in ice-cold preparation medium consisting of modified Eagle's medium with 1% L-glutamine at pH 7.4 under sterile conditions. The brains were sliced horizontally (350 μm) with a vibratome (Pelco, Redding, CA), beginning at the ventral surface. Slices containing the hippocampi were cultured on 6-well cell-culture plates (Corning) in cultivation medium

containing 50% modified Eagle's medium, 25% Hank's balanced salt solution (Gibco), 25% normal horse serum (Gibco), 2% glutamine, 10 μg/ml insulin–transferrin–sodium selenite supplement (Boehringer Mannheim, Mannheim, Germany), 2.64 mg/ml glucose (Braun, Melsungen, Germany), 0.1 mg/ml streptomycin, 100 U/ml penicillin, and 0.8 mg/ml ascorbic acid, and incubated in a humidified atmosphere at 37°C, 5% $O_2$. Death of neurons was assessed by addition of propidium iodide (PI; 1 μg/ml; Sigma) to the medium for 30 min at the end of the incubation period, as previously described (Wolf et al., 2002). Excess PI was then washed off with cultivation medium, and the slices were prepared for microscopy and visualized. To quantify neuronal death in the OHSCs, the intensity of PI staining in the measured area of each slice was recorded with Image-Pro Plus software (v4.5). The effects of different treatments were compared using Student's two-tailed *t*-test.

***(xvii) Application of microglia on hippocampal slices.*** Cultures of treated or untreated microglia were washed with cultivation medium to remove all traces of the tested reagents, kept on ice for 15 min, and shaken at 350 rpm for 20 min at room temperature. Microglia were removed from the flasks and immediately co-cultured ($1\times10^5$ cells/slice/ml) with freshly prepared OHSCs for 24 h. Neutralizing ãIGF-I Abs (10 μg/ml) were applied on slices without treatment or were co-incubated with naïve microglia or microglia treated with IL-4.

***(xviii) Immunocytochemistry.*** Cultured microglia on coverslips were fixed in 4% paraformaldehyde (pH 7.4) for 20 min at room temperature and then stored in PBS at 4°C pending examination. Specimens were treated with a permeabilization/blocking solution containing 10% FCS, 2% bovine serum albumin, 1% glycine, and 0.1% Triton X-100 (Sigma-Aldrich). Primary antisera were applied for 1 h in a humidified chamber at room temperature. For labeling of microglia we used either mouse anti-ED1 antibody (1:200; Serotec) or FITC-conjugated *Bandeiraea simplicifolia* isolectin B4 (I-B4, 1:50; Sigma-Aldrich). To detect expression of cell-surface MHC-II we used mouse anti-MHC-II Abs (1:50; IQ Products, Groningen, The Netherlands). Expression of IGF-I was detected by

goat anti-IGF-I Abs (1:10–1:100; R&D Systems). Secondary antibodies were all purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) and used at a dilution of 1:250. Control sections (not treated with primary antibody) were used to distinguish specific staining from staining of nonspecific antibodies or autofluorescent components. All experiments were independently replicated at least three times. The observer who analyzed the results was blinded to the identity of the experimental groups. Immunoreactivities in microglia were quantified in digital photomicrographs (1024×1024 pixels, 0–255, RGB) of the fluorescence obtained by FITC staining (for I-B4), PI staining (for nuclei), and Cy5 staining (for MHC-II) or Cy3 (for IGF-I) using a Zeiss LSM 510 confocal laser scanning microscope (40× magnification). Sixteen fields (each 230×230 μm) per slide for each treatment (n = 4). Immunoreactivities were quantified using Image-Pro Plus 4.5 (Media Cybernetics, Silver Spring, MD). The values obtained were divided by the number of microglia in the same image that were double-labeled by PI and I-B4. All non-microglial cells (<2–5%) were excluded from the calculation. A two-tailed Student's t-test was used to compare the relative staining intensities of treatment and control groups.

***(xix) Terminal dUTP nick-end labeling.*** Cultured microglia were subjected to terminal dUTP nick-end labeling (TUNEL; R&D Systems), used as a marker of apoptosis. Cells were double-labeled with ED1-antibodies for 1 h at room temperature in a humidified chamber, rinsed three times with 0.05% Tween-20 in PBS, and incubated with the secondary antibody Cy5-conjugated goat anti-mouse IgG (1:200; Jackson ImmunoResearch) for 1 h at room temperature. The cells were then labeled with PI (0.25% vol/vol; Molecular Probes, Eugene, OR). Preparations were mounted and covered for examination under a Zeiss laser-scanning confocal microscope (LSM510).

***(xx) RNA purification, cDNA synthesis, reverse transcription PCR, and real-time quantitative PCR analysis.*** Cells were lysed with TRI reagent (MRC, Cincinnati, OH), and total cellular RNA was purified from lysates using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Residual genomic DNA was removed during the purification process by incubation with RNase-free DNase (Qiagen). RNA was stored in Rnase-free water (Qiagen) at –80 °C. RNA (1 μg) was converted to cDNA using SuperScript II (Promega, Madison, WI), as recommended by the manufacturer. The cDNA mixture was diluted 1:5 with PCR-grade water. We assayed the expression of specific mRNAs using semi-quantitative reverse transcription PCR (RT–PCR) and real-time quantitative PCR (Q-PCR) with selected gene-specific primer pairs. Ii (CD74; invariant chain), CIITA (class II MHC transactivator), STAT1 (signal transducer and activator of transcription), and IGF-I (insulin-like growth factor 1) were designed using OLIGO v6.4 (Molecular Biology Insights, Cascade, CO). The primers used were: for the Ii, sense, 5'-ACCAGCGCGACCTCATCTCT-3', antisense 5'-AGCAAGGGAGTAGCCATGCG-3' (target size, 297 bp); STAT1, sense 5'-GGTGAACCCGACTTCCATGC-3', antisense 5'-AGCTCCATCGGTTCTGGTGC-3' (target size, 221 bp); CIITA, sense 5'-TTTCAGGGCCTCCTTGAGTGAC-3', antisense 5'-AGCAAGGGAGTAGCCATGCG-3' (target size, 373 bp); TNF-α, sense 5'-AGGAGGCGCTCCCCAAAAAGATGGG-3', antisense 5'-GTACATGGGCTCATACCAGGGCTTG-3' (target size, 551 bp); (E) IGF-I, sense 5'-CAGGCTCCTAGCATACCTGC-3', antisense 5'-GCTGGTAAAGGTGAGCAAGC-3' (target size, 244 bp); β-actin, sense 5'-TTGTAACCAACTGGGACGATATGG-3', antisense 5'-GATCTTGATCTTCATGGTGCTAGG-3' (target size, 764 bp). RT–PCR reactions were carried out using 1 μg of cDNA, 35 nmol of each primer, and ReadyMix PCR Master Mix (ABgene, Epsom, UK) in 30-μl reactions. PCR reactions were carried out in an Eppendorf PCR system with cycles (usually 25–30) of 95°C for 30 s, 60°C for 1 min, 72°C for 1 min, and 72°C for 5 min, and then kept at 4°C. β-actin mRNA was used as an internal standard for the amount of cDNA synthesized. PCR products were subjected to agarose gel analysis and visualized by ethidium bromide staining. Signals were quantified using a Gel-Pro analyzer 3.1 (Media Cybernetics,

Silver Spring, MD). In all cases, one product was observed with each primer set, and the observed product had an amplicon size that matched the size predicted from published cDNA sequences.

Q-PCR reactions were performed with a high-speed thermal cycler (LightCycler; Roche Diagnostics, Indianapolis, IN), and the product was detected by FastStart Master SYBR Green I (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. The amplification cycle was 95°C for 10 s, 60°C for 5 s, and 72°C for 10 s. Sequences for the primers of CIITA and Ii were as described above. Melting curve analysis confirmed that only one product was amplified.

**EXAMPLE 5. Microglia activated by aggregated β-amyloid, like LPS-activated microglia, destroy neural tissue.**

Studies by our group demonstrated that T cells directed against self-antigens residing in the damaged CNS promote neuronal recovery (Moalem et al., 1999; Butovsky et al., 2001; Schwartz and Kipnis, 2003; Shaked et al., 2004a). It was suggested that such T cells modify the activity of microglia. This beneficial effect was found to be correlated with expression of class II major histocompatibility proteins (MHC-II) and the costimulatory molecule B7-2 (Bechmann et al., 2001; Butovsky et al., 2001), both characteristic properties of antigen-presenting cells (APCs). Impairment of this T cell-mediated neuroprotective mechanism might lead to further degeneration (Schori et al., 2001; Schori et al., 2002). Accordingly, we postulated that microglia might be the cells which, depending on how they interpret their milieu, can exert a beneficial or a destructive effect on the CNS tissue.

To test this hypothesis, we measured the neuronal losses in rat organotypic hippocampal slice cultures (OHSCs) in an *ex-vivo* bioassay to study the survival of neural tissue following a change in the extracellular environment. Excision and slicing of the hippocampus cause loss of neural tissue, the extent of which is dependent on the nature of the surrounding milieu (Wolf et al., 2002). Tissue loss is assessed by incorporation of propidium iodide (PI), which stains dead cells without

distinguishing between neurons and non-neurons. This protocol allows assessment of the general state of devastation of the damaged tissue in the context of its external conditions.

We first examined whether aggregated β-amyloid 1-40 (hereafter referred to as $A\beta_{(1-40)}$), a self-compound which in its aggregated form is associated with neuronal degeneration, causes microglia to become cytotoxic. As a contril we used the bacterial wall-associated compound LPS known to be associated with inflammation, (Fig. 1). Naïve microglia were incubated 24 h with LPS (100 ng/ml) or with $A\beta_{(1-40)}$ (50 μM), or were left untreated. They were then washed well and co-cultured with the hippocampal slices. After 24 h the slices were washed and stained with PI (see Material and Methods). Figs. 8A and 8B show that incubation with untreated microglia resulted in some neural cell death beyond that caused by slicing of the tissue. In comparison, loss of neural tissue in the slices incubated with LPS-activated microglia or with microglia activated by $A\beta_{(1-40)}$ was dramatic. To get an insight to the nature of the interactions between LPS or $A\beta_{(1-40)}$ and microglial cells we examined the signal transduction pathway at the transcription and protein levels. Such an examination revealed that both LPS and $A\beta_{(1-40)}$ had activated a pathway unrelated to the family of signal transducers and activators of transcription (STAT1), a characteristic activator of adaptive immunity (Stark et al., 1998), and did not activate a pathway leading to MHC-II expression, which normally involves expression of Ii and CTIIA (Stumptner-Cuvelette and Benaroch, 2002). Interestingly, both LPS and $A\beta_{(1-40)}$ up-regulated the expression of tumor necrosis factor (TNF)-α (Fig. 8 C).

**EXAMPLE 6. Adaptive immunity instructs microglia to become protective.**

Next we examined whether microglia pre-activated by cytokines (associated with adaptive immunity) characteristic of Th1 or Th2 develop a phenotype that differs from that obtained upon activation with LPS or $A\beta_{(1-40)}$ (Figs. 8A, B). Fig. 9A shows that IFN-γ, a characteristic Th1 cytokine, rendered the microglia

protective at low doses (1–20 ng/ml) but cytotoxic at higher doses, and that the characteristic Th2 cytokine IL-4 endowed the microglia with a protective phenotype at all concentrations tested. As in the above experiments (Figs. 8A, B), microglia that were not activated prior to co-culture with the hippocampal slices had a weak negative effect on neural loss (Fig. 9A). I Analysis of the phenotypes of the signal transduction activated by IFN-γ and by IL-4 revealed that both cytokines activated the STAT1 pathway and genes associated with MHC-II expression (Fig. 9B). Moreover, whereas IL-4 down-regulated the production of TNF-α, IFN-γ (20 ng/ml) caused a slight though transient increase in TNF-α expression (Fig. 9C). Expression of Ii and CTIIA transcripts was activated by both IL-4 and IFN-γ, especially the latter (Fig. 9C).

To verify the above results at the protein level, we assayed MHC-II expression by immunocytochemical staining (Fig. 10A). LPS and $A\beta_{(1-40)}$ both failed to induce expression of MHC-II. We therefore first compared the expression of MHC-II by IL-4-activated and IFN-γ-activated microglia as a function of time. After 12 h of exposure, microglial MHC-II expression was induced earlier by IFN-γ than by IL-4 (Fig. 10B). After 24 h of exposure, however, microglial MHC-II expression induced by IL-4 was significantly higher than that induced by IFN-γ (Fig. 10B). TUNEL staining for apoptotic cells ruled out the possibility that this failure of expression was attributable to microglial death (Fig. 10C).

Having shown that microglia activated by $A\beta_{(1-40)}$, similarly to LPS-activated microglia, behave differently from microglia activated by IFN-γ or IL-4, we were interested in determining whether early exposure of the microglia to these cytotoxic agents commits the microglia irreversibly to destructive activity. To that end we assayed the expression of MHC-II at the transcript and protein levels (Fig 4). The results showed that when microglia were exposed to LPS, they failed to express Ii and CIITA transcripts if subsequently exposed to IFN-γ (Figs. 11A, B). A somewhat different picture was seen with exposure to $A\beta_{(1-40)}$ and then to IFN-γ. Here too Ii was hardly expressed, STAT1 was not affected and CIITA expression

was reduced as was tested by quantitative PCR analysis (Figs. 11A, B, C). As in the case of LPS, however, secretion of TNF-α was uncontrolled and was as high as with LPS. It thus appears that early exposure to cytotoxic agents is hardly reversible by IFN-γ (Figs. 11 A–C). Examination of the cells at the protein level (MHC-II) revealed a similar picture (Figs. 11 D and E). Interestingly, the addition of ãTNF-α boosted the IFN-γ-induced increase in MHC-II expression (Fig. 11 D).

We also examined whether the effect of early exposure to LPS or to $A\beta_{(1-40)}$ can be overcome by IL-4. Fig. 11 F shows that IL-4 partially overcame the negative effect of LPS or $A\beta_{(1-40)}$, leading to expression of MHC-II.

**EXAMPLE 7. IL-4 instructs microglia to become protective via induction of IGF-I.**

The fact that IL-4 but not IFN-γ overcame to some extent the effects of both LPS and $A\beta_{(1-40)}$ prompted us examine whether early exposure to these cytokines would endow the microglia with added resistance to the negative effect of a cytotoxic agent such as LPS. To this end, we first assayed the protective activity of cytokine-activated microglia in hippocampal slices. We found that IL-4, and (though to a significantly smaller extent) IFN-γ, could overcome the cytotoxic effect of LPS (Fig. 12A). These results, coupled with the above findings, led us to propose that the mechanism of protection developed by IL-4 differs from that developed by IFN-γ. Moreover, the superior protective effect of IL-4 to that of IFN-γ shown in our *ex-vivo* assay of hippocampal slices, as well as the ability of IL-4 to overcome the negative effect of LPS, encouraged us to further investigate the activity of the IL-4-activated microglia.

A striking difference in the gene array analysis of microglia activated by MBP-reactive T cells and microglia treated with aggregated $A\beta_{(1-40)}$ relates to the expression of IGF-I, which we found to be up-regulated relative to untreated microglia after exposure of the microglia to MBP-activated T cells and down-regulated by their exposure to aggregated $A\beta_{(1-40)}$ (data not shown). We therefore analyzed the expression, at the mRNA level, of IGF-I by microglia after their

exposure to IL-4, IFN-γ, and LPS. RT–PCR analysis revealed that IL-4, unlike IFN-γ, is a strong inducer of IGF-I expression (Fig. 12B). As the figure shows, IL-4 caused a dramatic increase in microglial expression of IGF-I, whereas IFN-γ caused a slight reduction. Moreover, brief exposure (2 h) of the microglia to IL-4 prior to their exposure to LPS was sufficient to obtain stronger microglial expression of IGF-I than that seen in microglia exposed only to LPS. Analysis of IGF-I expression at the protein level supported the results at the mRNA level by showing that IL-4 induced IGF-I expression, unlike IFN-γ (which down-regulated it slightly) and LPS (which completely blocked it). IL-4-treated microglia were able to maintain the expression of IGF-I to some extent (by approximately 3 fold of that seen in untreated microglia), even if the microglia were exposed to LPS after their exposure to IL-4 ($P < 0.01$, Fig. 12C). A similar picture was obtained with aggregated $A\beta_{(1-40)}$; this agent down-regulated microglial expression of IGF-I, but subsequent exposure of the microglia to IL-4 enabled the cells to overcome the down-regulation effect (Fig. 13).

Having demonstrated that IL-4 induces microglial production of IGF-I, we examined whether this neurotrophic factor contributes to the IL-4-induced neuroprotective microglial phenotype. Microglia were cultured for 24 h in the presence or absence of IL-4. The cells were then thoroughly washed and added to OHSCs, with or without ãIGF-I. The same antibodies were added directly to the OHSCs to rule out their direct effect on neural tissue survival. Addition of ãIGF-I significantly weakened the neuroprotective effect of the IL-4-activated microglia ($P<0.05$; Fig. 14).

## Discussion

### Microglia phenotype is a reflection of their environment

Adaptive immunity, through its major characteristic cytokines IFN-γ and IL-4, was found here to induce a protective phenotype in microglia, shown by the

observed effect of the activated microglia on brain hippocampal slices. The range of IFN-γ concentrations capable of inducing the neuroprotective microglial phenotype was narrow, whereas IL-4 induced a protective phenotype over a wider range of concentrations and also had the effect of up-regulating IGF-I. Exposure of microglia to $A\beta_{(1-40)}$, like their exposure to LPS, resulted in a cytotoxic microglial phenotype. Cytotoxicity was associated with up-regulation of TNF-α. The cytotoxicity induced by LPS or by $A\beta_{(1-40)}$ could be partly overcome by pretreatment of the microglia with IL-4, but not with IFN-γ. Examination of the signal transduction pathways activated by the tested compounds disclosed that protection, unlike cytotoxicity, was accompanied by activation both of STAT1 and of proteins associated with MHC-II expression.

**Cytotoxicity of aggregated β-amyloid mimics LPS**

The results of this study suggest that an insult by $A\beta_{(1-40)}$ causes microglia to adopt a destructive phenotype reminiscent of their lethal response to invading microorganisms (as exemplified by their response to LPS). This phenotype is one that the CNS can hardly tolerate. Such microglia are not only detrimental to neural tissue, but they also evade regulation by the adaptive immune system, whose function—according to findings by our group (Schwartz et al., 2003)—is to fight off such toxicity.

Microglia activated by LPS produce excessive levels of nitric oxide (NO; causing oxidative stress) and TNF-α, as well as other cytotoxic elements, which lead to a spiral of worsening neurotoxicity (Boje and Arora, 1992). Depending upon the sequence of signaling, LPS can either inhibit or stimulate the IFN-γ-induced expression of MHC-II by macrophages; it is inhibitory if given before or together with IFN-γ, but stimulatory if given later (Sicher et al., 1995). TNF-α, like LPS, inhibits IFN-γ-induced expression of MHC-II by microglia if NO is produced, but stimulates it in the absence of NO formation (Sicher et al., 1995).. Thus, TNF-α apparently antagonizes the effect of IFN-γ on the regulation of microglial MHC-II

expression. This is consistent with findings that TNF-α-treated mice show decreased expression of Ia on peritoneal macrophages and that treatment with TNF-α *in vivo* inhibits the ability of IFN-γ to induce MHC-II expression by these macrophages (Melhus et al., 1991). Moreover, TNF-α suppresses IFN-γ-induced MHC-II expression on macrophages by destabilizing CIITA mRNA (Han et al., 1999).

Our finding that aggregated-β-amyloid induced in microglia a similar effect to that of LPS is in line with a study *in vitro* showing that β-amyloid fibrils, like stimulation by LPS, increase microglial/monocytic production of TNF-α and NO (Combs et al., 2001). Other studies have shown that the induction of TNF-α mRNA by β-amyloid is dependent on the fibrillary state of the peptide; β-amyloid that is rendered nonfibrillar has no effect on induction of TNF-α mRNA (Yates et al., 2000). Moreover, a recent study showed that the LPS receptor (CD14), a key receptor for innate immunity proteins, interacts with fibrils of Alzheimer amyloid peptide. Neutralization of this receptor with anti-CD14 antibodies, or genetic deficiency of CD14, significantly reduced the microglial activation induced by this peptide and attenuated the toxic microglial effect on neurons (Fassbender et al., 2004). The link between TNF-α and the stage of β-amyloid aggregation is in line with our contention that certain self-compounds, after undergoing changes in conformation, structure, or state of aggregation, impose on brain-resident immune cells a phenotype which is reminiscent of that needed to fight off exogenous microorganisms and which is inappropriate for the adaptive immunity needed to fight off unfriendly self-compounds (Schwartz et al., 2003). It thus seems that infective self-agents can escape immune regulation by preventing the relevant microglia from acquiring an APC phenotype.

**IL-4-induces IGF-I production**

The protective effect of the microglia induced *ex vivo* by IL-4 was superior to that induced by any other tested agent. It should be emphasized that the bioassay

used here to determine microglia activity unlike the situation *in vivo*, is not affected by infiltrating immune cells. Nevertheless, analysis of IL-4-activated microglia revealed that it was characterized by down-regulated production of TNF-α and up-regulation of IGF-I.. IL-4 was reported to be beneficial in hippocampal cultures, where it markedly increased the numbers of astroglia and microglia (Araujo and Cotman, 1993). This neuroprotective effect was suggested to result from inhibition of the microglial production of TNF-α and nitric oxide (Chao et al., 1993). IL-4, even when administered 30 min before exposure of microglia to LPS, inhibits LPS-induced microglial production of inducible nitric oxide synthase (iNOS) and TNF-α (Kitamura et al., 2000). Microglia express the IL-4 receptor in culture, but RT–PCR analysis showed that they do not express the IL-4 cytokine itself (Suzumura et al., 1994). Furthermore, CD14 (the LPS receptor) is down-regulated by IL-4 in microglia by inhibition of CD14 mRNA (Becher et al., 1996).

The neuroprotective trophic factor IGF-I plays a pivotal role in regulating inflammatory events in the brain (Dore et al., 1997), and appears to interact *in vivo* with pro-inflammatory cytokines that are produced in the CNS (Dantzer et al., 1999). A decrease in serum IGF-I is correlated with neurodegenerative conditions (Busiguina et al., 2000). Furthermore, insulin modulates clearance of β-amyloid (Gasparini et al., 2001), an effect antagonized by TNF-α (Carro et al., 2002). In addition TNF-α, at concentrations that do not directly reduce neuronal survival, strongly interferes with the ability of IGF-I to increase the activity of PI3-kinase, which is essential for the IGF-I-induced promotion of neuronal survival (Venters et al., 2001). IGF-I prevents the acute effect of glutamate-mediated toxicity on oligodendrocytes *in vitro* (Ness and Wood, 2002) and inhibits apoptosis of mature oligodendrocytes during primary demyelination (Mason et al., 2000). TNF-α and IGF-I are potentially induced in the same areas of the CNS in response to neurodegenerative conditions such as ischemia. Early observations of TNF-α in the CNS focused on the ability of this cytokine to inhibit the release and action of neurotransmitters.

## Adaptive immunity and neurodegenerative disorders

Class II MHC genes, needed for the presentation of antigens required for T-cell activation, are regulated primarily at the transcriptional level, and the class II transactivator CIITA is the "master control factor" of class II MHC transcription (Rohn et al., 1996). In the present study, priming of microglia with aggregated $A\beta_{(1-40)}$ or LPS significantly reduced their expression of CIITA upon subsequent stimulation with IFN-γ. We found, moreover, that transcripts encoding Ii are down-regulated in both LPS-primed and $A\beta_{(1-40)}$-primed microglia, resulting in their lack of cell-surface expression of MHC-II. To the best of our knowledge, this is the first report of self-compounds that can affect intracellular regulatory mechanisms in a way that causes them to evade adaptive immunity.

The results of this study suggest that exogenous microorganisms and toxic self-compounds operate through similar intracellular signaling cascades. Outside the CNS, defense against invading microorganisms is universally viewed as the outcome of successful adaptation. Within the CNS, however, such defense must be considered as a maladaptation. This is because, as we discovered here, microglia that encounter self-compounds which are harmful to neural tissue, and which fail to express MHC-II, are not only toxic to the tissue but also evade the local interaction with T cells (adaptive immunity) needed to control their activity in a way that allows them to protect the tissue against local threats (such as oxidative imbalance and cytotoxicity of neurotransmitters). It thus seems, paradoxically, that rendering the microglia cytotoxic precludes their participation in the adaptive immune response needed to rescue the tissue from the toxicity that they themselves have caused. In line with this notion is the reported observation that accumulation of β-amyloid and plaque formation continue as long as there is no expression of MHC-II in the damaged area (Gordon et al., 2002). According to this view, vaccination with β-amyloid, even if it successfully evokes a systemic T cell response, is unlikely to be fully effective in achieving T cell-dependent activation of the microglia (Avidan et al., 2004). This is because microglia that are exposed to $A\beta_{(1-40)}$, and are

subsequently encountered by $A\beta_{(1-40)}$-specific T cells that home to the lesion site, are incapable of expressing MHC-II and hence of presenting the β-amyloid antigen to T cells (unlike in the case where specific antigens at the site of a lesion are presented to homing T cells by the relevant APCs) (Mizrahi et al., 2002).. It should be stressed that such impairment of the physiological T cell-mediated response does not necessarily contradict reports that antibodies directed against β-amyloid have a beneficial effect in reducing β-amyloid-associated plaque (Janus et al., 2000).

The findings of this study also lead us to suggest that a common feature of neurodegenerative diseases might be interaction between the causative self-compounds and the microglia, and hence that therapy should be based on modulating this interaction rather than suppressing it. We further suggest that self-agents associated with deposition diseases are harmful to the tissue, not only on account of their inherent toxicity, but also because they lead to impairment of the adaptive immune response that their cytotoxicity evokes. According to this view, it is likely that the ability of the CNS to withstand the consequences of an acute injury depends on the ability to acquire, assisted by adaptive immunity, the "correct" microglial phenotype.

Taking the results of the present *in vitro* study at face value, it might be argued that Th2 cells (with their characteristic cytokine IL-4) are superior to Th1 cells (with IFN-γ) for neural protection. This might not, however, be the case *in vivo*, in light of the following: (a) in the face of a threatening situation (e.g., after acute axonal injury), expression of a neuroprotective effect by the microglia is dependent on their rapid response (before they can commit to a destructive phenotype), and this can be achieved by IFN-γ (Kipnis et al., 2002a); (b) compared to microglia activated by IL-4, IFN-γ-activated microglia are more proficient at glutamate uptake (Shaked et al., 2004b), an essential activity for the dialogue between T cells and APCs; and (c) Th1 cells, unlike Th2 cells, are likely to help recruit additional immune cells when critically needed. In light of all the above, and based on the results of the present study, it seems reasonable to suggest that a well-controlled adaptive immunity, initiated by a transient and controlled Th1 phase

(Shaked et al., 2004a) and followed by a complementary Th2 response, might enable the CNS to maximize its potential for fighting off local threats. Moreover, the present results might support a new approach, based on vaccination, in the search for an immune-based therapy, as they suggest that the relevant antigen to be used for recruiting T cells to a site of injury is not the toxic self-compound itself (e.g., β-amyloid), but a non-encephalitogenic self-antigen (Hauben et al., 2001) that is not toxic and resides at the damaged site.

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## CLAIMS

1. A method for treating a neurodegenerative disorder or disease in which there is accumulation of misfolded and/or aggregated proteins, excluding prion-related diseases, said method comprising administering to an individual in need an agent selected from the group consisting of (i) Copolymer 1, (ii) a Copolymer 1-related peptide, (iii) a Copolymer 1-related polypeptide, and (iv) T cells activated with (i), (ii) or (iii).

2. A method in accordance with claim 1, wherein said neurodegenerative disease or disorder is selected from the group consisting of Huntington's disease, Alzheimer's disease and Parkinson's disease.

3. A method in accordance with claim 2, wherein said agent is Copolymer 1.

4. A method in accordance with claim 2, wherein said agent is a Copolymer 1-related peptide or a Copolymer 1-related polypeptide.

5. A method in accordance with claim 2, wherein said agent is T cells which have been activated by Copolymer 1.

6. A method for reducing disease progression, and/or for protection from neurodegeneration and/or protection from glutamate toxicity in a patient suffering from a neurodegenerative disease or disorder selected from the group consisting of Huntington's disease, Alzheimer's disease and Parkinson's disease, which comprises immunizing said patient with an agent selected from the group consisting of (i) Copolymer 1, (ii) a ~~Copolymer 1-related peptide~~, (iii) ~~a~~ Copolymer 1-related polypeptide, and (iv) T cells activated with (i), (ii) or (iii).

7. A method for reducing disease progression, and/or for protection from neurodegeneration and/or protection from glutamate toxicity in a patient suffering from a neurodegenerative disease or disorder selected from the group consisting of Huntington's disease, Alzheimer's disease and Parkinson's disease, which comprises administering to said patient in need a therapeutically effective amount of

an agent selected from the group consisting of (i) Copolymer 1, (ii) a Copolymer 1-related peptide, (iii) a Copolymer 1-related polypeptide, and (iv) T cells activated with (i), (ii) or (iii).

8. A method for reducing disease progression, and/or for protection from neurodegeneration and/or protection from glutamate toxicity in a patient suffering from Alzheimer's disease, which comprises administering to an individual in need thereof an effective amount of an agent selected from the group consisting of (i) Copolymer 1, (ii) a Copolymer 1-related peptide, (iii) a Copolymer 1-related polypeptide, and (iv) T cells activated with (i), (ii) or (iii).

9. A pharmaceutical composition for treatment of a neurodegenerative disorder or disease in which there is accumulation of misfolded and/or aggregated proteins, excluding prion-related diseases, comprising a pharmaceutically acceptable carrier and an active agent selected from the group consisting of (i) Copolymer 1, (ii) a Copolymer 1-related peptide, (iii) a Copolymer 1-related polypeptide, and (iv) T cells activated with (i), (ii) or (iii).

10. A pharmaceutical composition according to claim 9, for reducing disease progression, and/or for protection from neurodegeneration, and/or protection from glutamate toxicity in a patient suffering from said neurodegenerative disorder or disease.

11. A pharmaceutical composition according to claim 9 or 10, wherein said neurodegenerative disease or disorder is selected from the group consisting of Huntington's disease, Alzheimer's disease and Parkinson's disease.

12. A pharmaceutical composition according to any one of claims 9 to 11, wherein said active agent is Copolymer 1.

13. Use of an active agent selected from the group consisting of (i) Copolymer 1, (ii) a Copolymer 1-related peptide, (iii) a Copolymer 1-related polypeptide, and (iv) T cells activated with (i), (ii) or (iii), for the manufacture of a pharmaceutical

composition for treatment of a neurodegenerative disorder or disease in which there is accumulation of misfolded and/or aggregated proteins, excluding prion-related diseases.

14. Use according to claim 13, wherein said neurodegenerative disease or disorder is selected from the group consisting of Huntington's disease, Alzheimer's disease and Parkinson's disease.

15. Use according to claim 13 or 14, wherein said active agent is Copolymer 1.

16. A method for treating or preventing neurodegeneration and cognitive decline and dysfunction associated with Huntington's disease, Alzheimer's disease orParkinson's disease, said method comprising administering to an individual in need an agent selected from the group consisting of (i) Copolymer 1, (ii) a Copolymer 1-related peptide, (iii) a Copolymer 1-related polypeptide, and (iv) T cells activated with (i), (ii) or (iii).

17. A method according to claim 16, wherein said active agent is Copolymer 1.

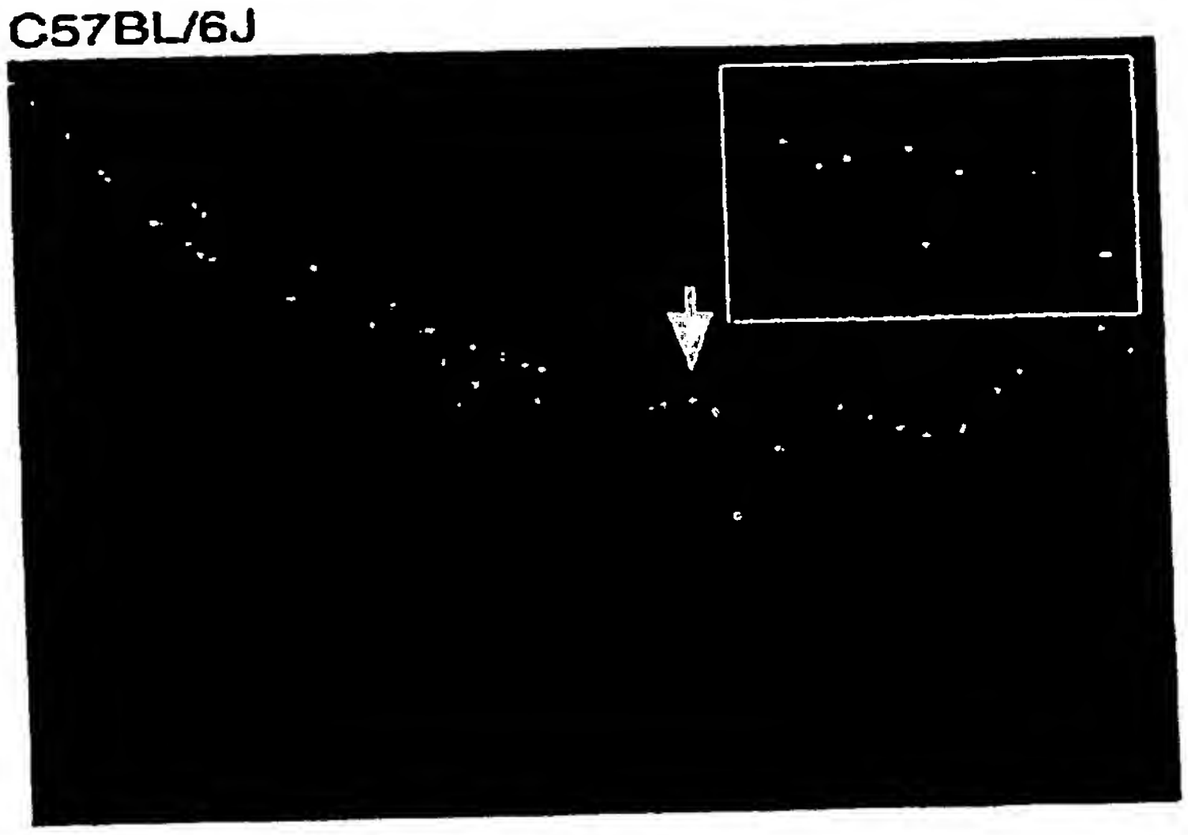
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**Fig. 1A**

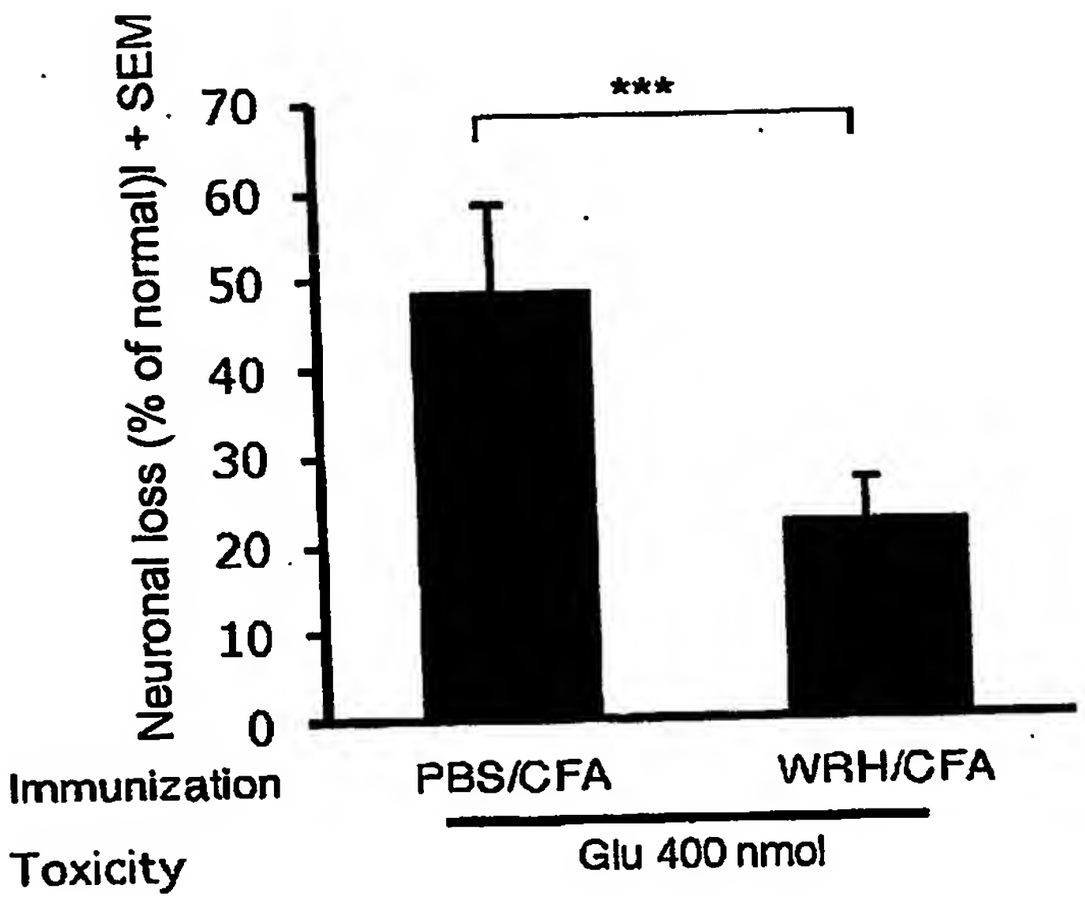
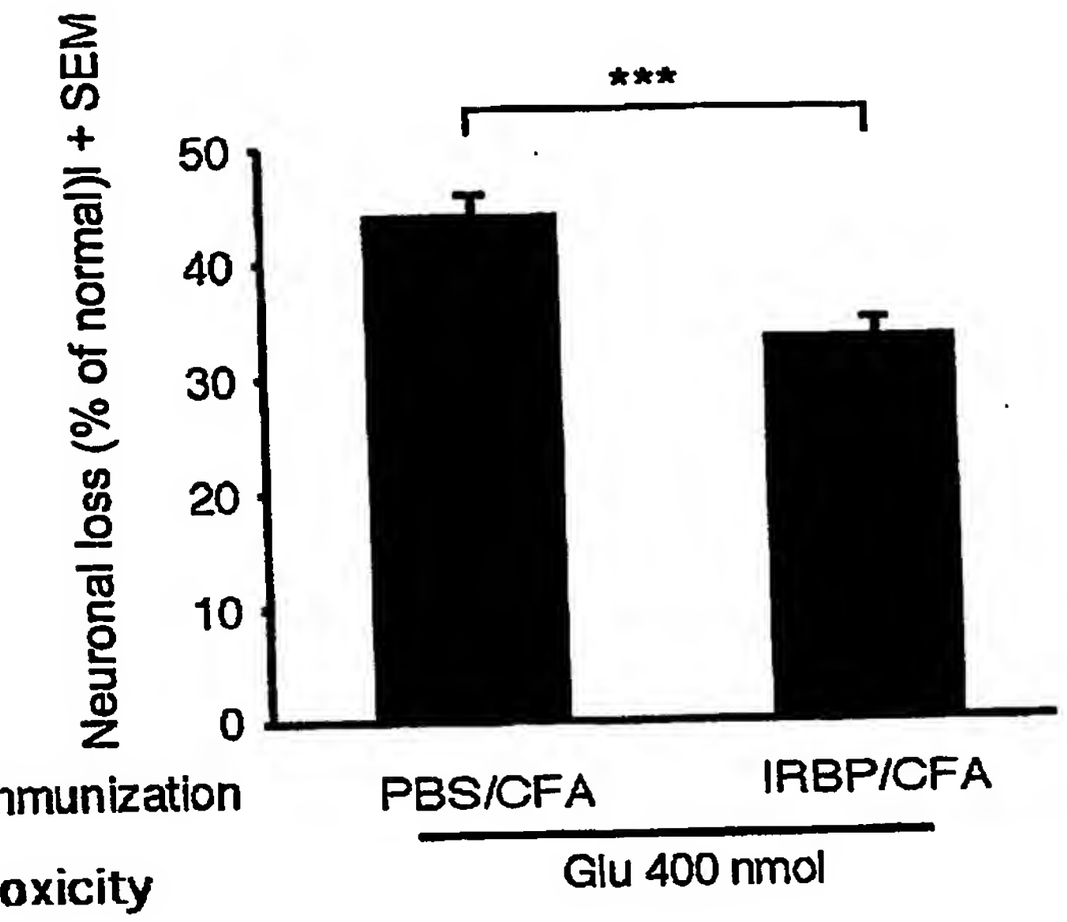

Neuronal loss (% of normal) + SEM
70
60
50
40
30
20
10
0
***
Immunization
PBS/CFA
WRH/CFA
Toxicity
Glu 400 nmol

**Fig. 1B**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Neuronal loss (% of normal)I + SEM
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30
20
10
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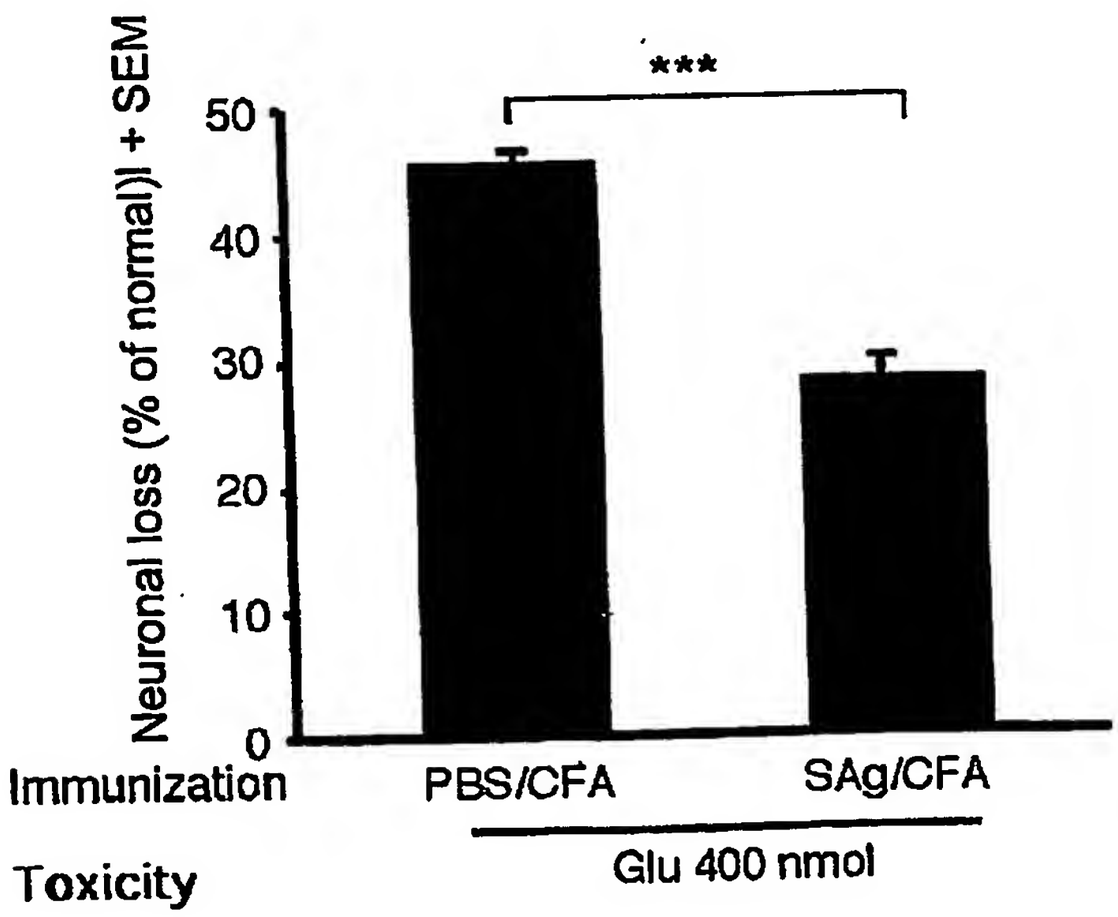
Immunization
PBS/CFA
IRBP/CFA
Toxicity
Glu 400 nmol

**Fig. 1C**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Neuronal loss (% of normal)I + SEM
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30
20
10
0
Immunization
PBS/CFA
SAg/CFA
Toxicity
Glu 400 nmol

**Fig. 1D**

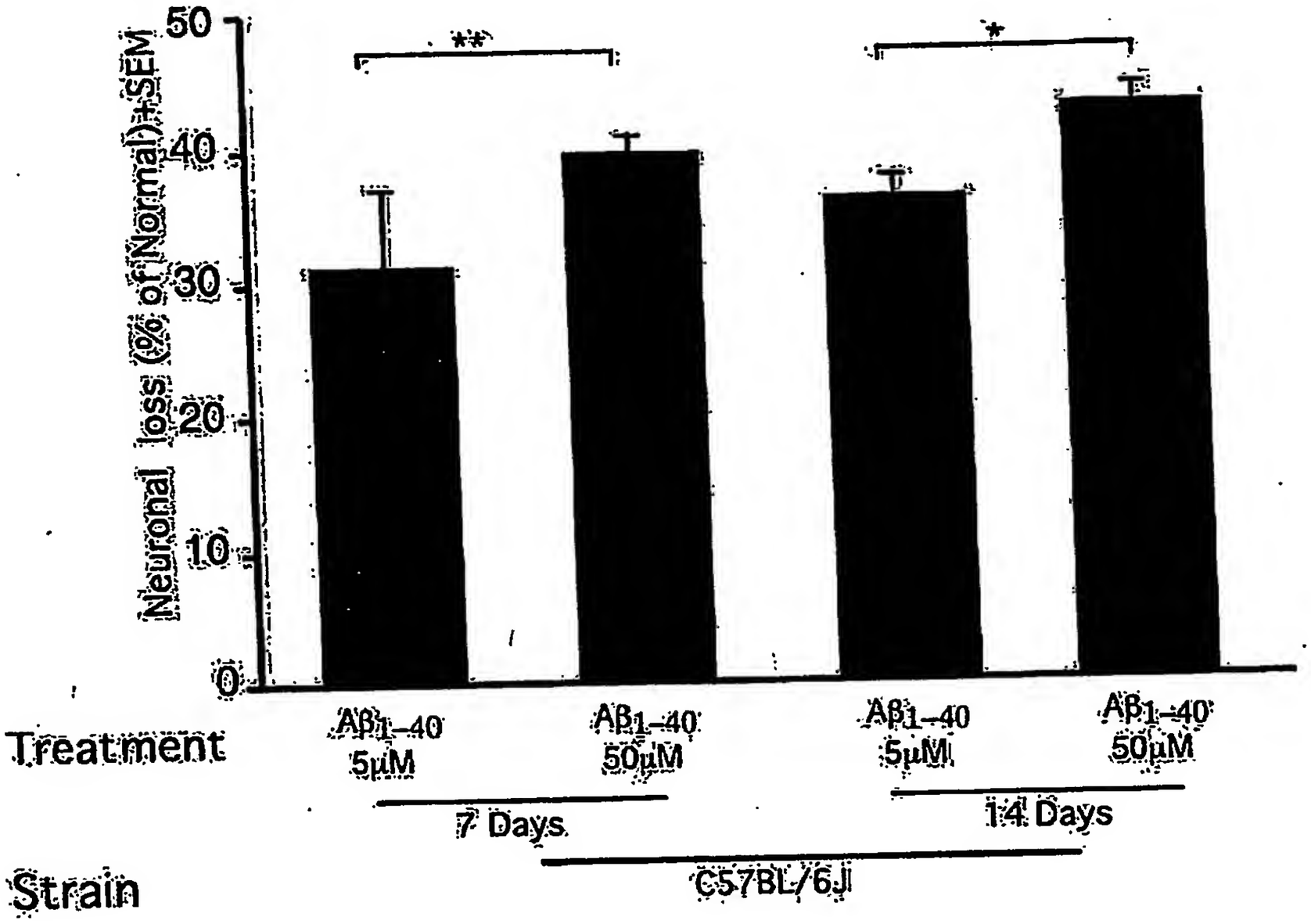



**Fig. 2A**

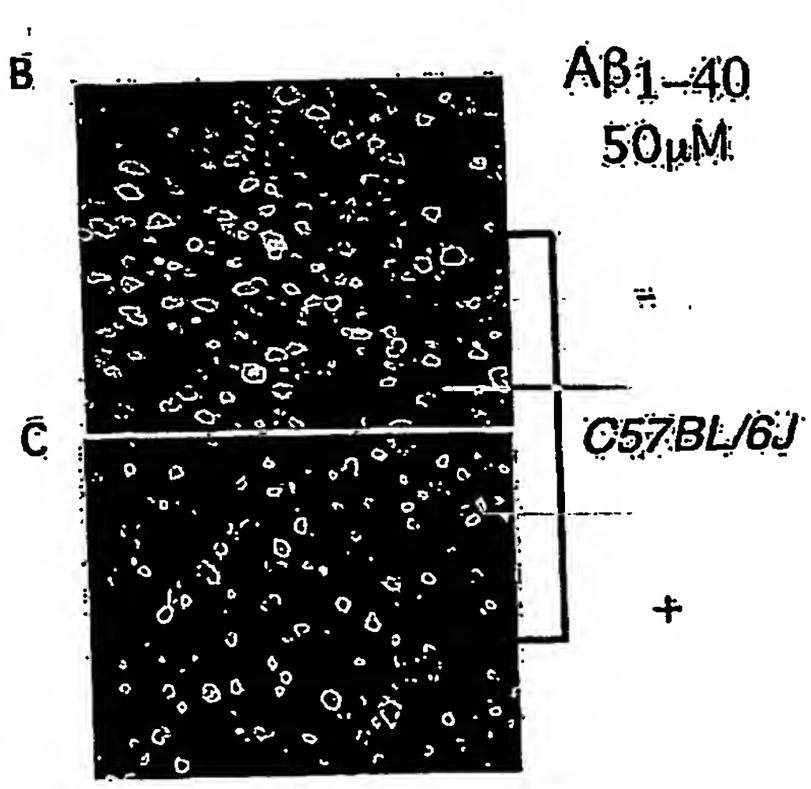



**Fig. 2B, 2C**

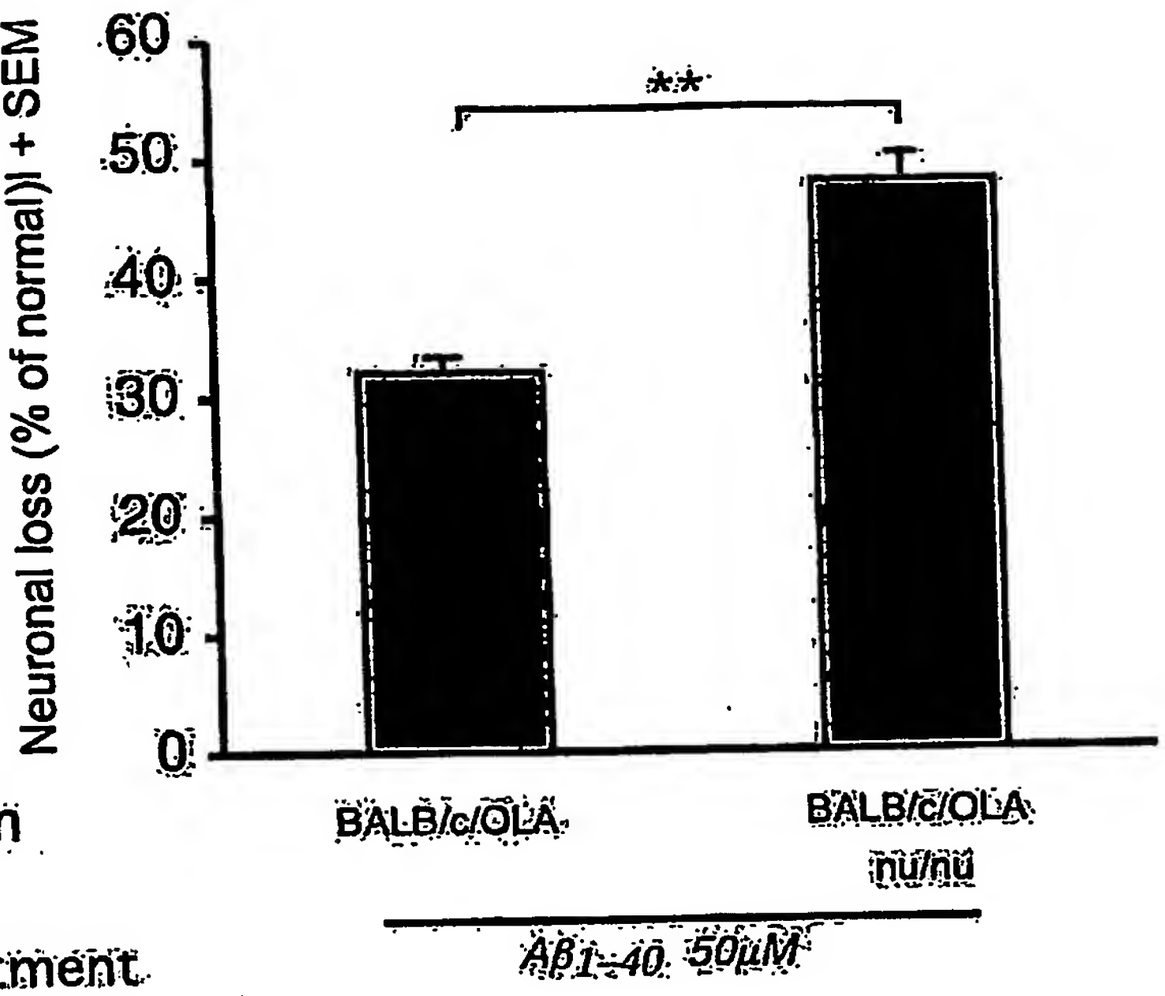



**Fig. 2D**

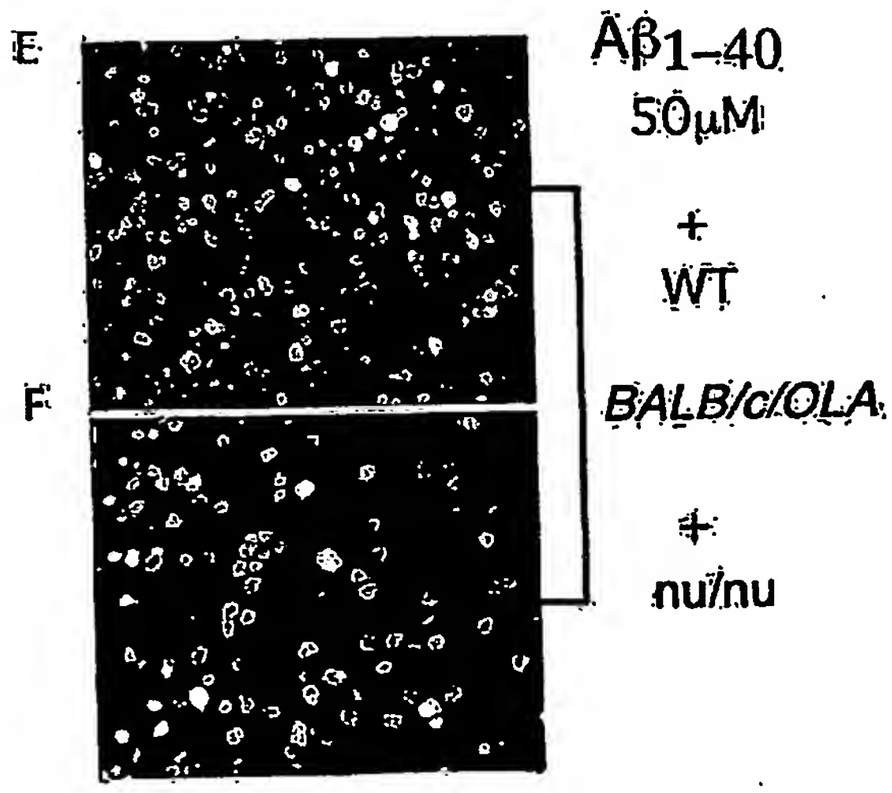
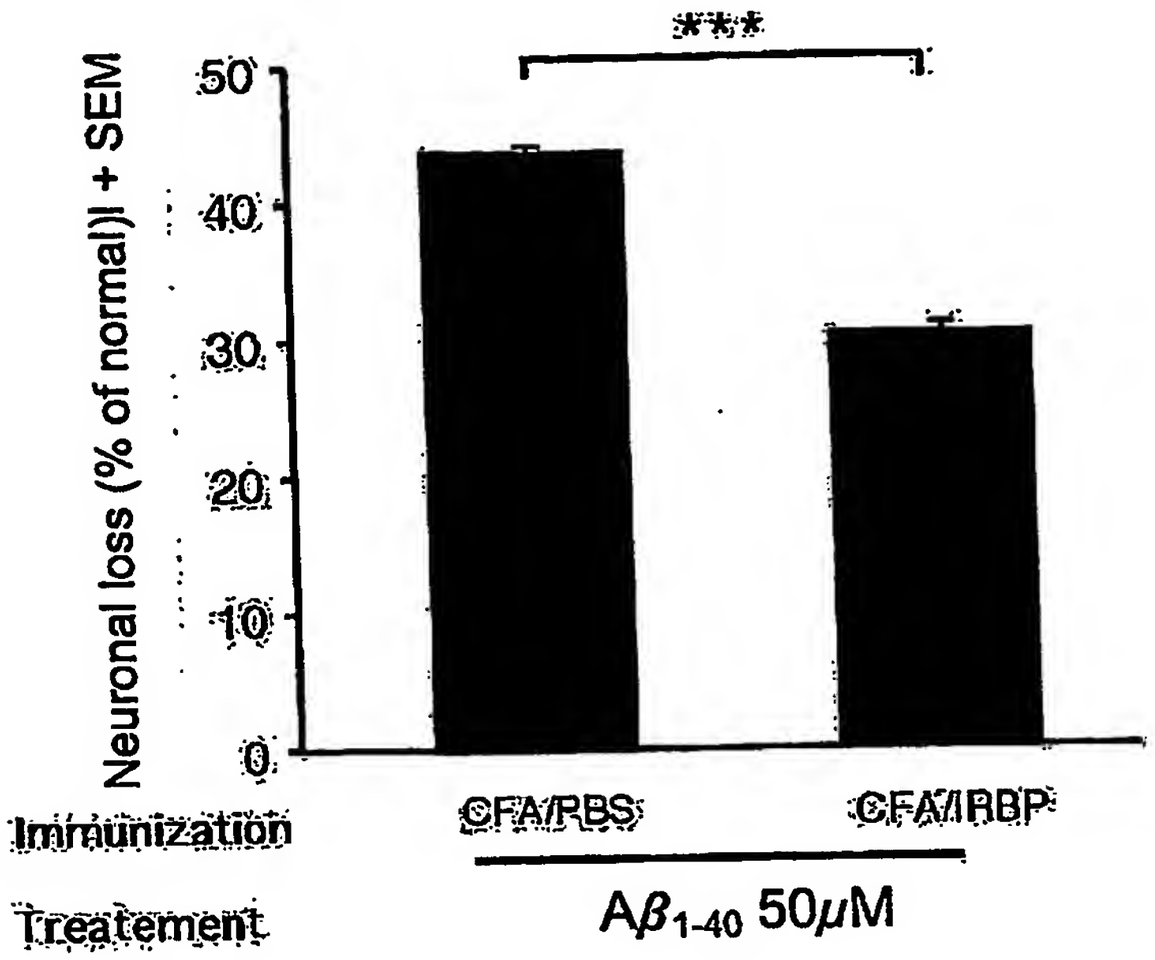



**Fig. 2E, 2F**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Neuronal loss (% of normal)I + SEM
50
40
30
20
10
0
CFA/PBS
CFA/IRBP
Immunization
Treatement
Aβ1-40 50μM

**Fig. 3A**

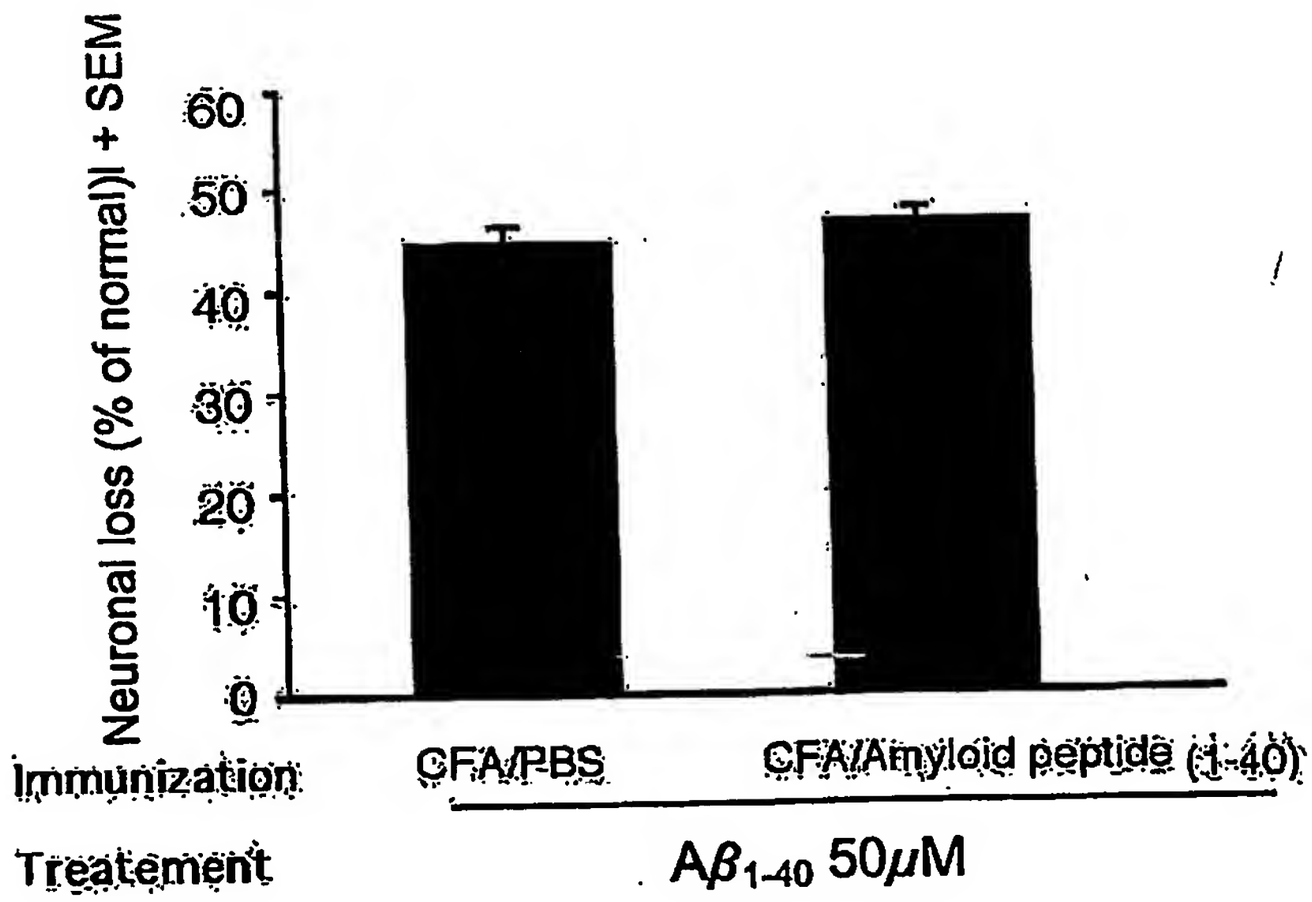
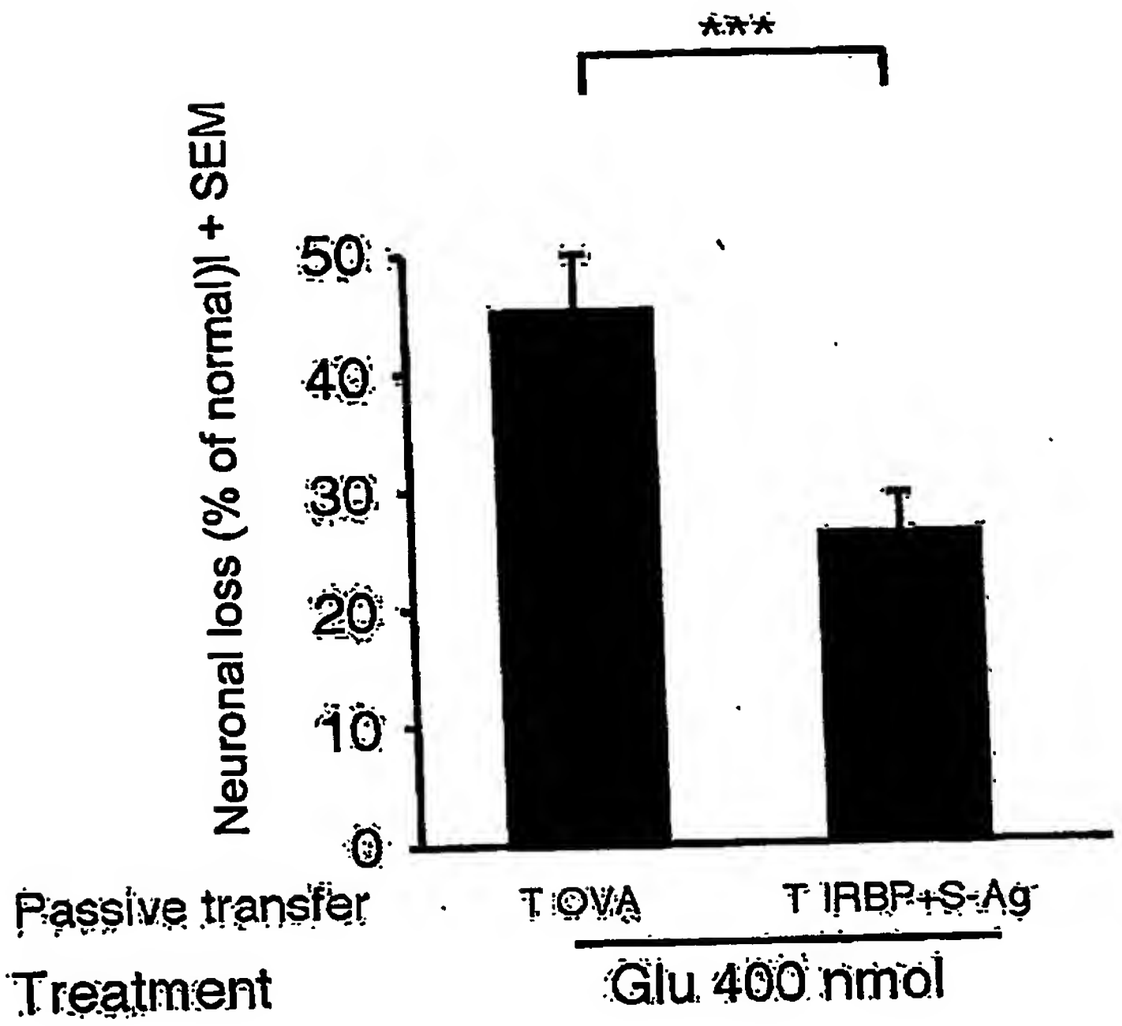

Neuronal loss (% of normal)I + SEM
60
50
40
30
20
10
0
CFA/PBS
CFA/Amyloid peptide (1-40)
Immunization
Treatement
Aβ1-40 50μM

**Fig. 3B**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Neuronal loss (% of normal)I + SEM
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40
30
20
10
0
Passive transfer
T OVA
T IRBP+S-Ag
Treatment
Glu 400 nmol

**Fig. 4A**

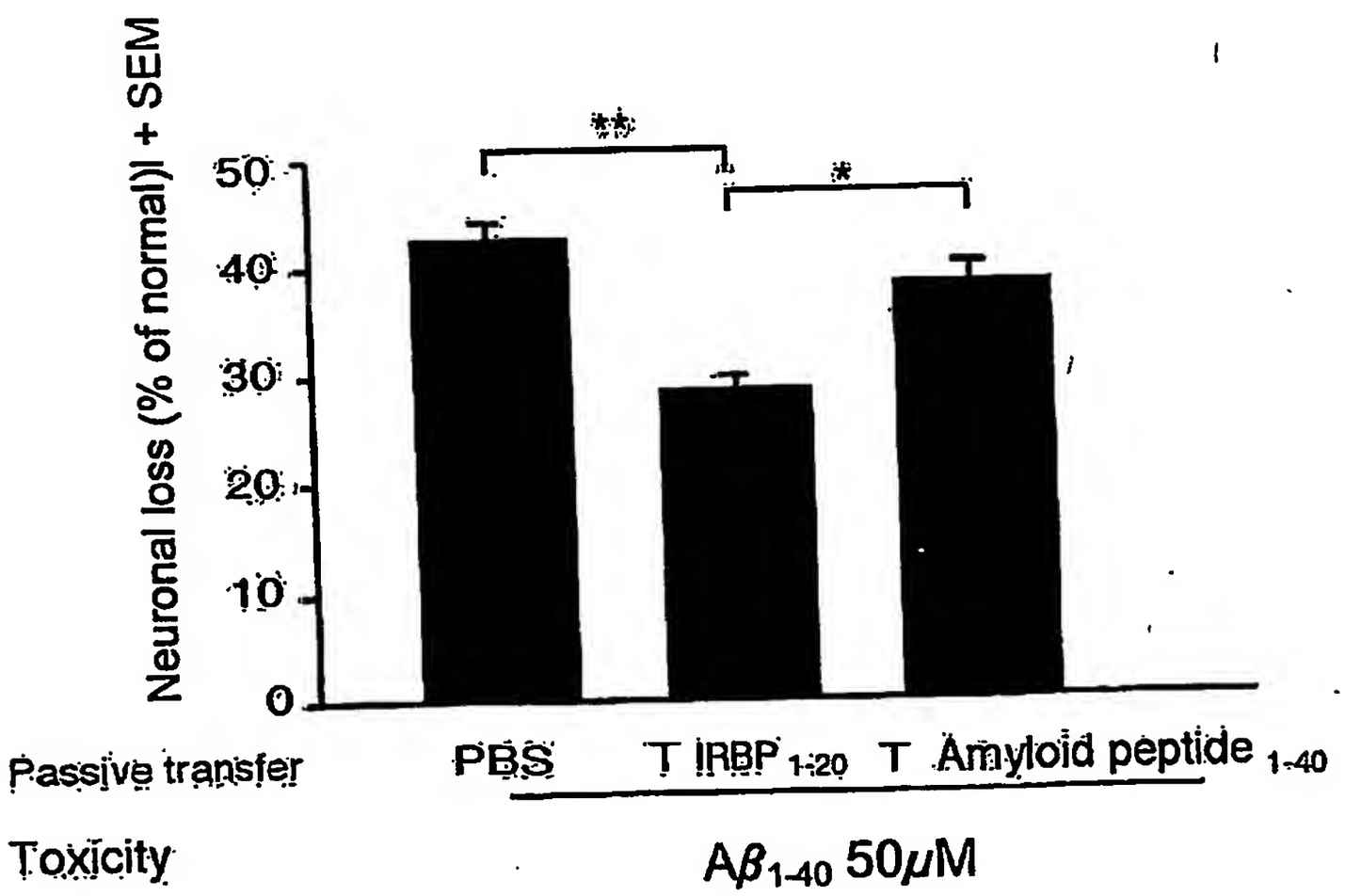

**
*
Neuronal loss (% of normal)I + SEM
50
40
30
20
10
0
Passive transfer
PBS
T IRBP 1-20
T Amyloid peptide 1-40
Toxicity
Aβ1-40 50μM

**Fig. 4B**

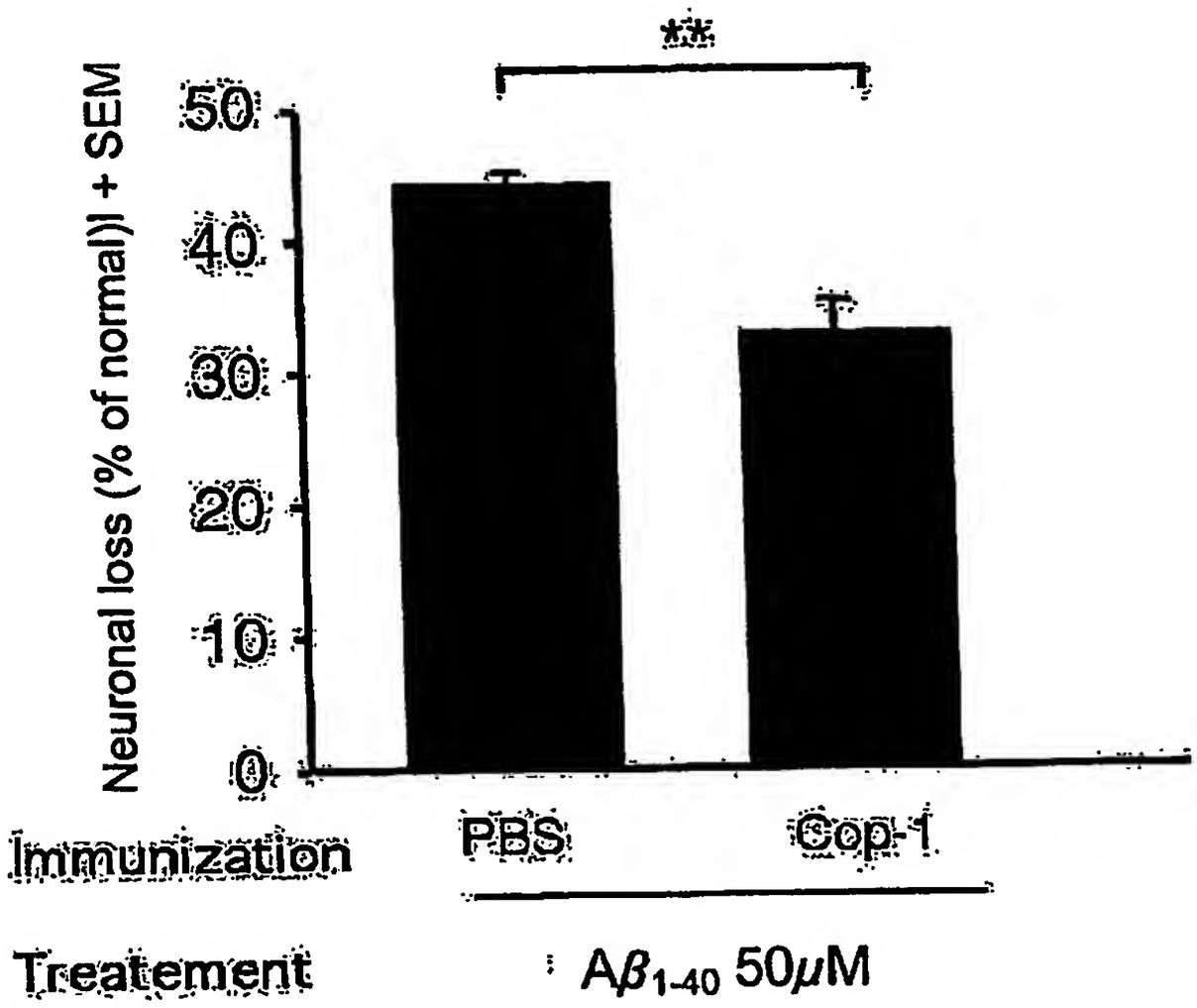

**
Neuronal loss (% of normal)l + SEM
50
40
30
20
10
0
Immunization
PBS
Cop-1
Treatement
Aβ1-40 50μM

**Fig. 5**

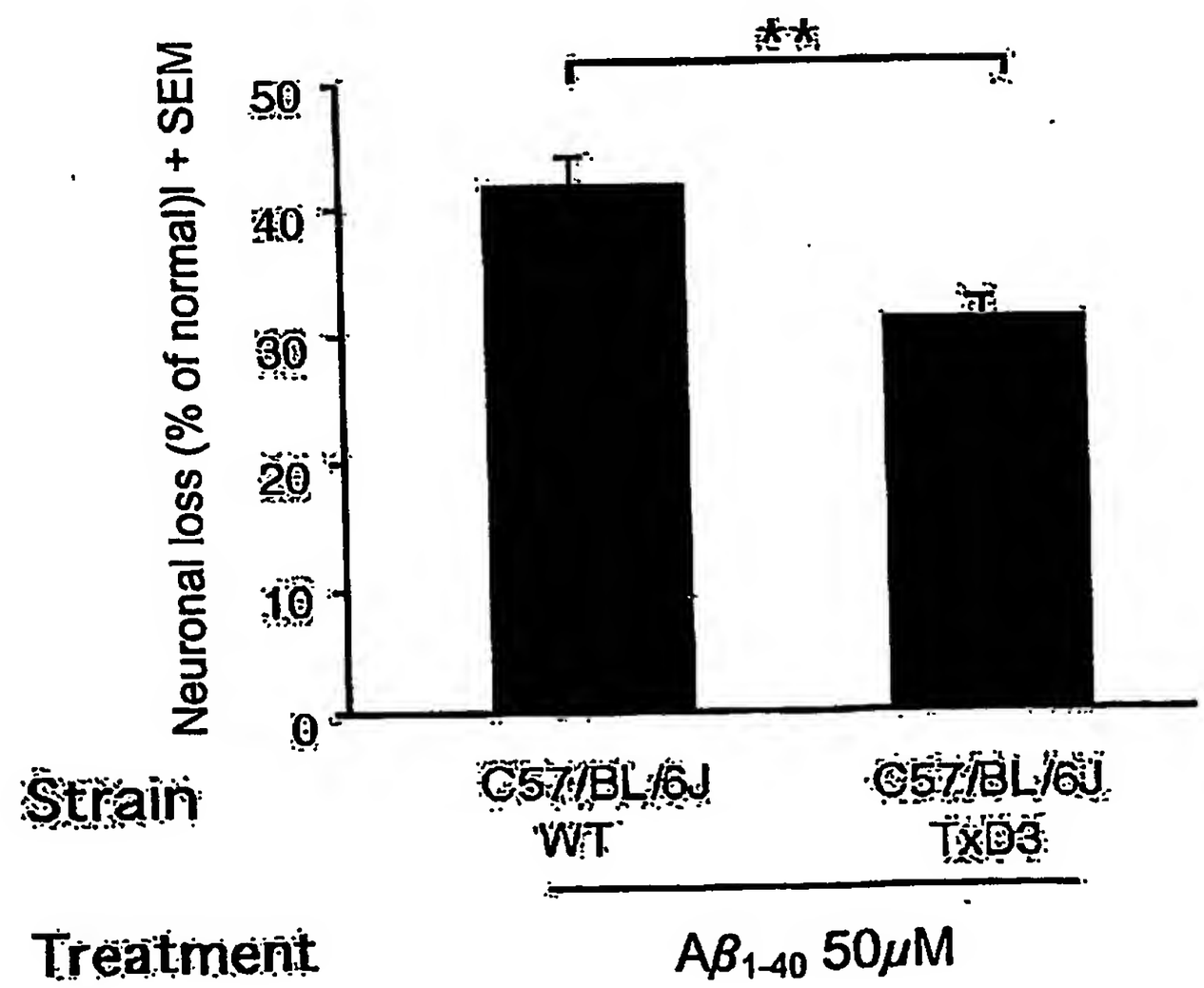

**
Neuronal loss (% of normal)l + SEM
50
40
30
20
10
0
Strain
C57/BL/6J
WT
C57/BL/6J
TxD3
Treatment
Aβ1-40 50μM

**Fig. 6A**

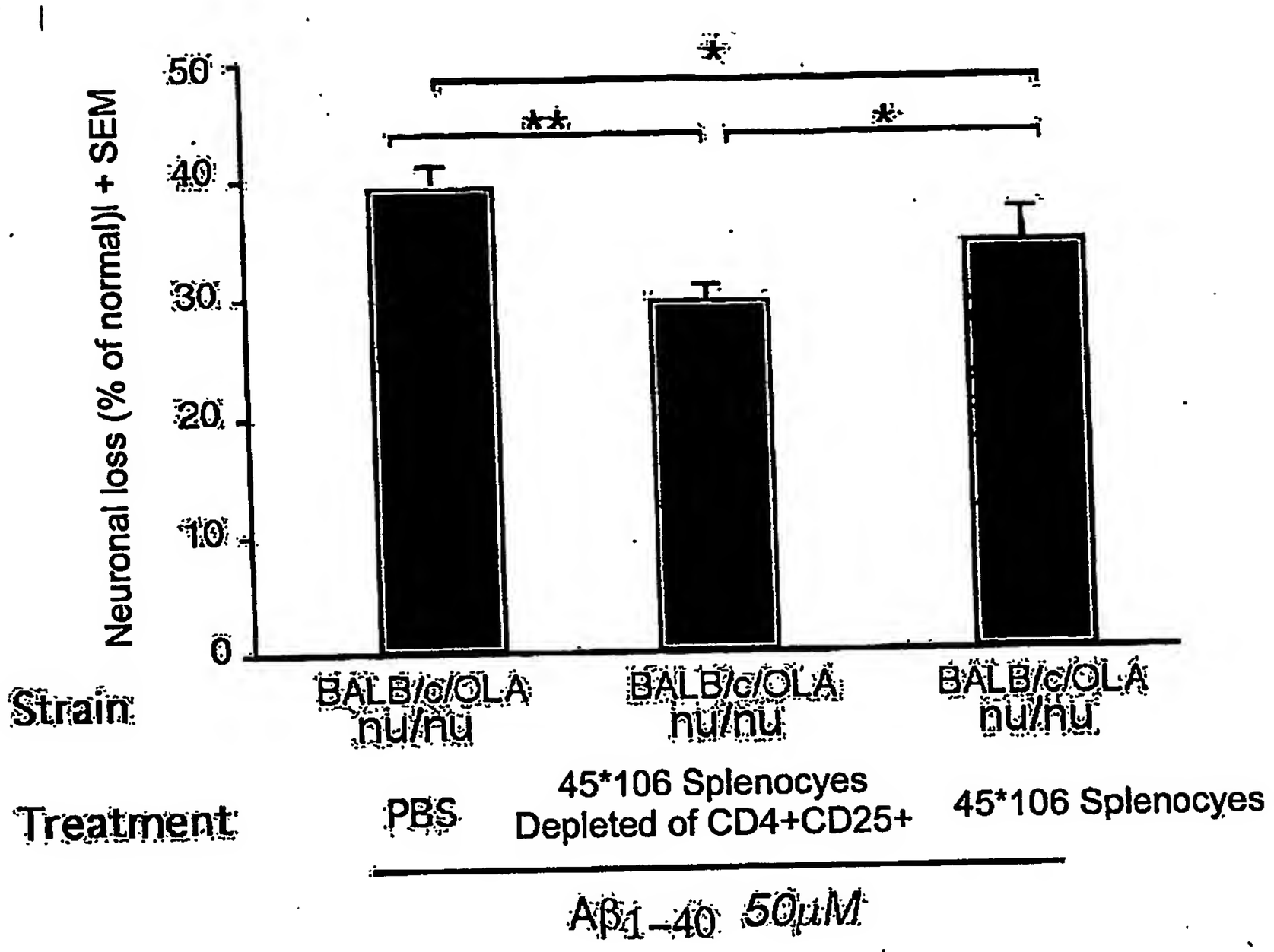

Neuronal loss (% of normal)I + SEM
50
40
30
20
10
0
*
**
*
Strain
BALB/c/OLA nu/nu
BALB/c/OLA nu/nu
BALB/c/OLA nu/nu
Treatment
PBS
45*106 Splenocyes Depleted of CD4+CD25+
45*106 Splenocyes
Aβ1-40 50μM

**Fig. 6B**

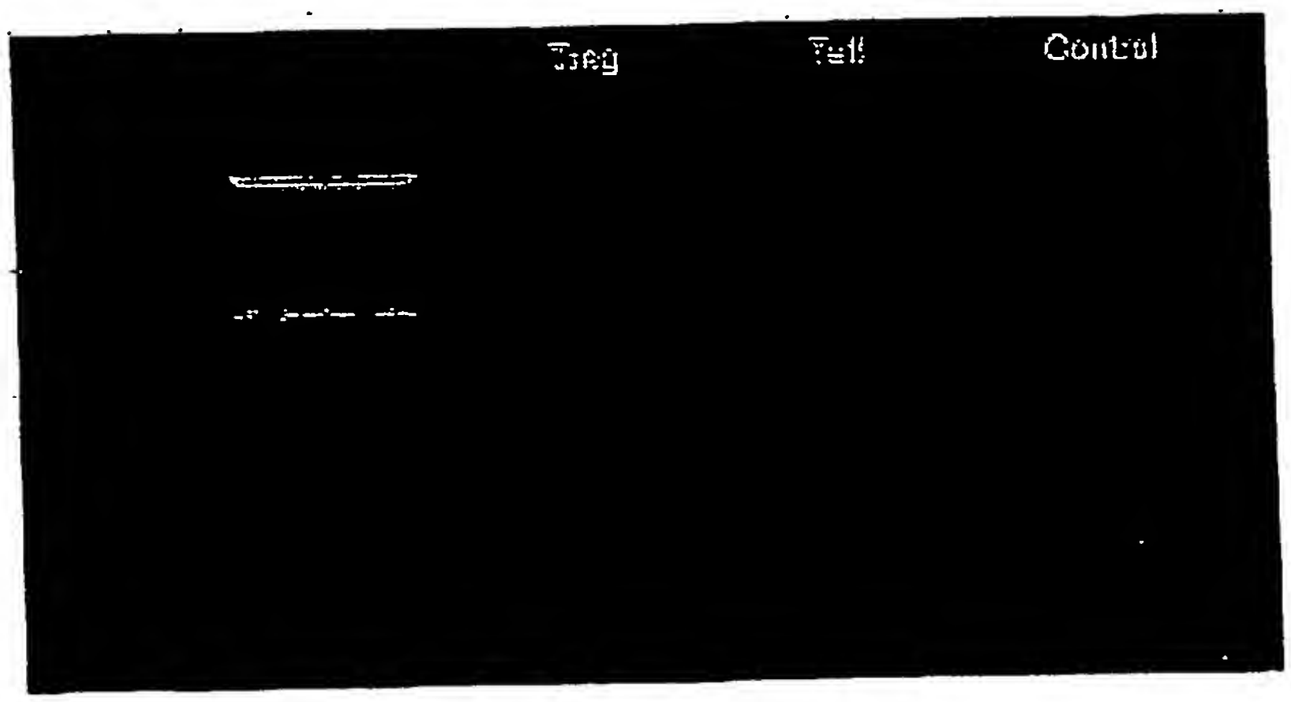

Treg
Teff
Control

**Fig. 6C**

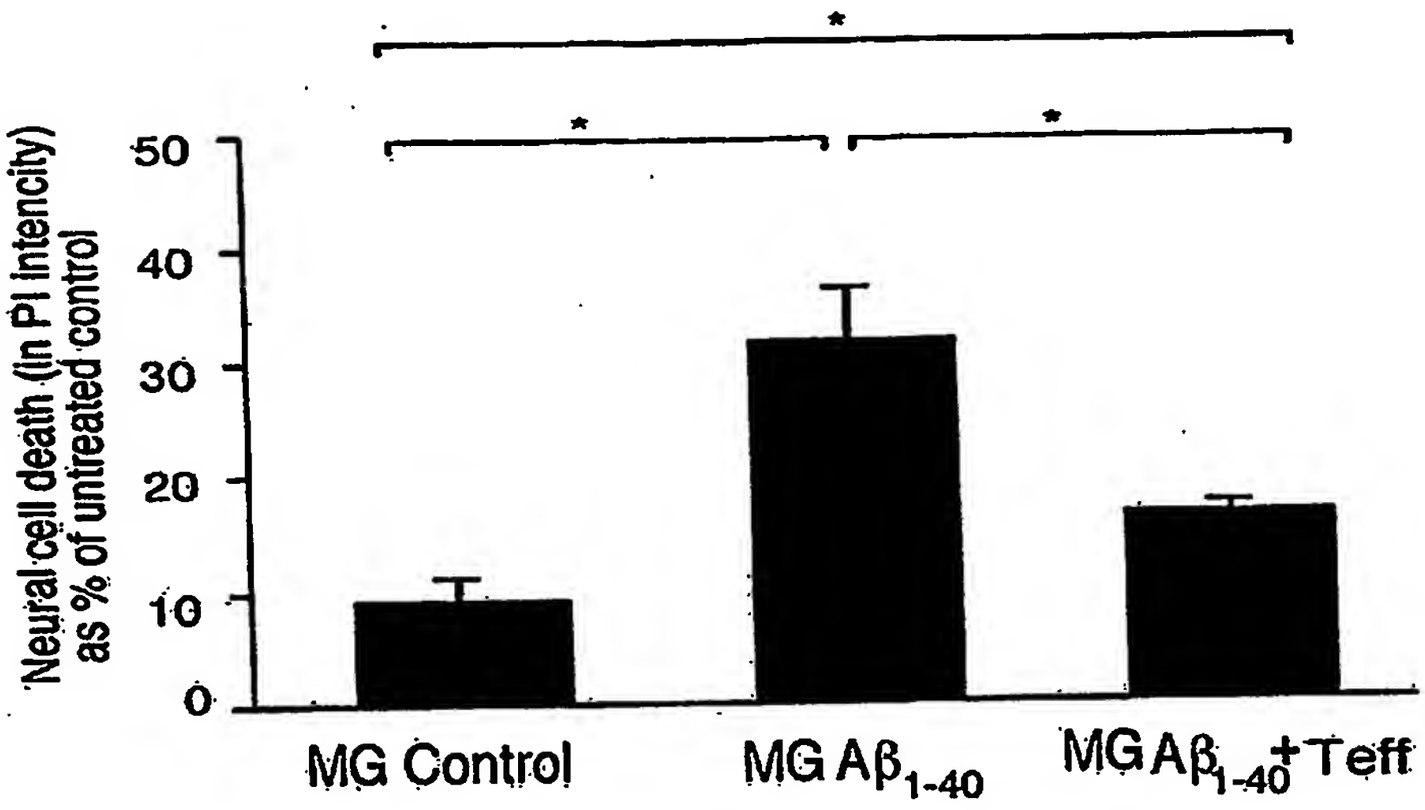

*
*
*
Neural cell death (ln PI intencity)
as % of untreated control
50
40
30
20
10
0
MG Control
MG Aβ1-40
MG Aβ1-40+Teff

**Fig. 7A**

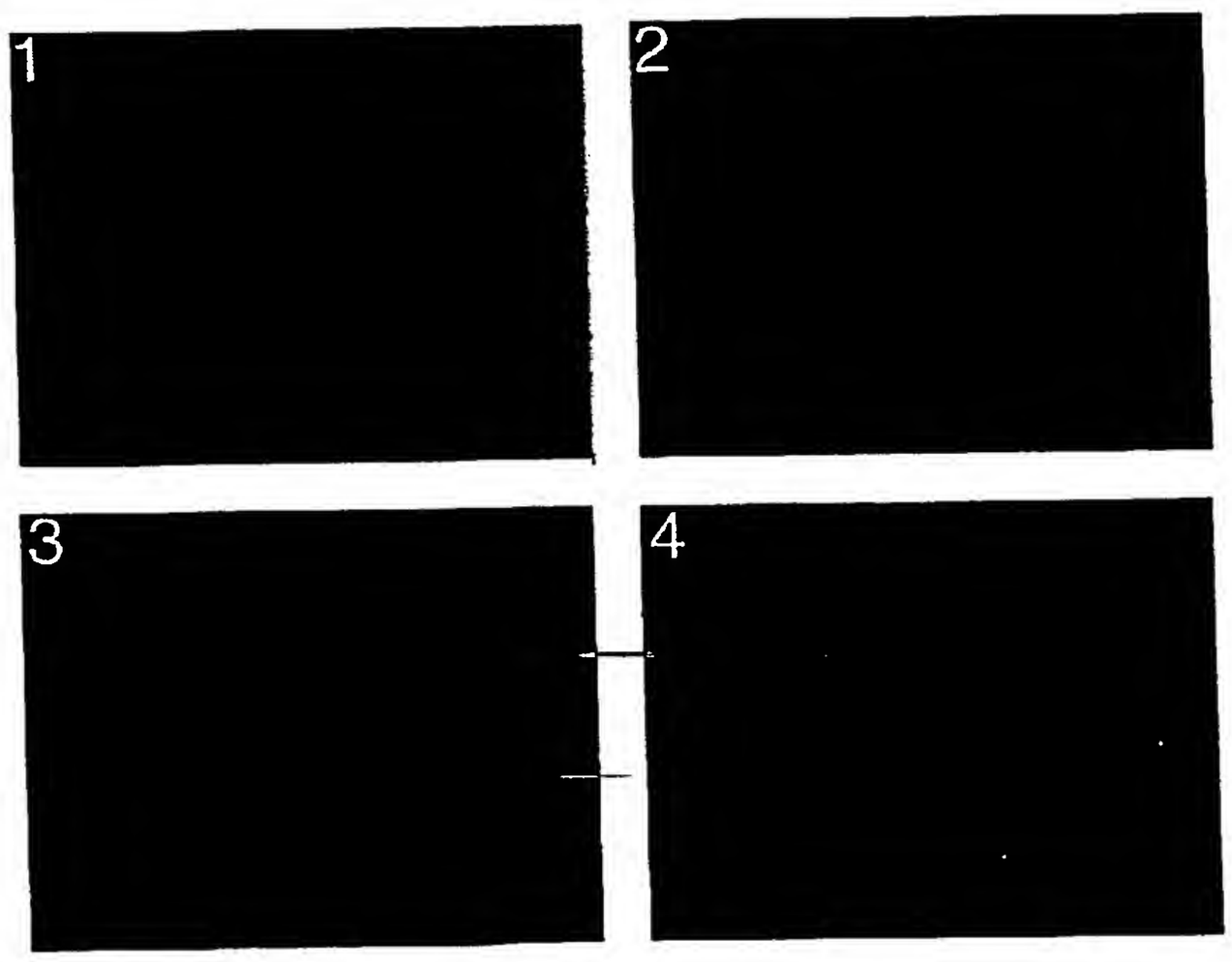

1
2
3
4

**Fig. 7B**

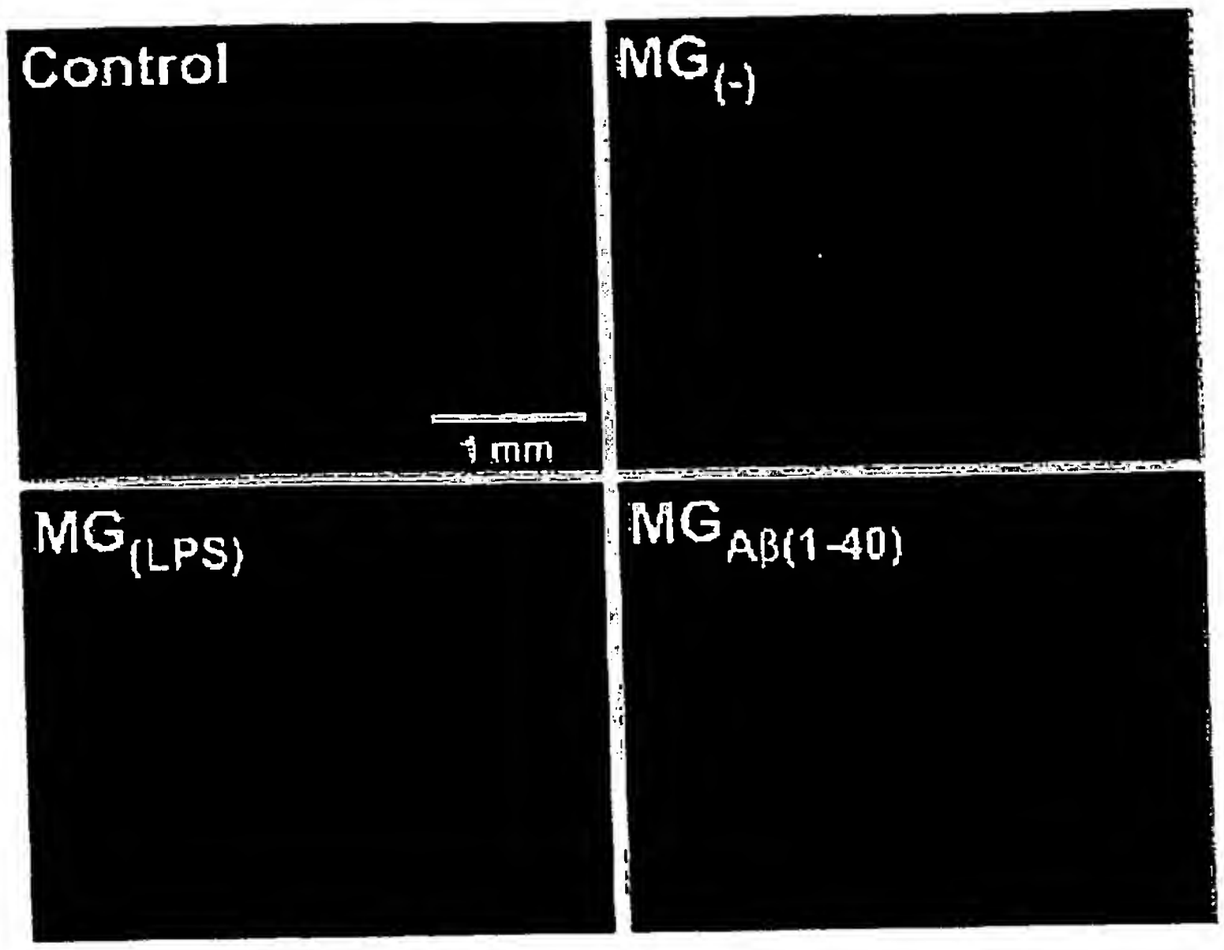

Control
1 mm
MG(-)
MG(LPS)
MGAβ(1-40)

Fig. 8A

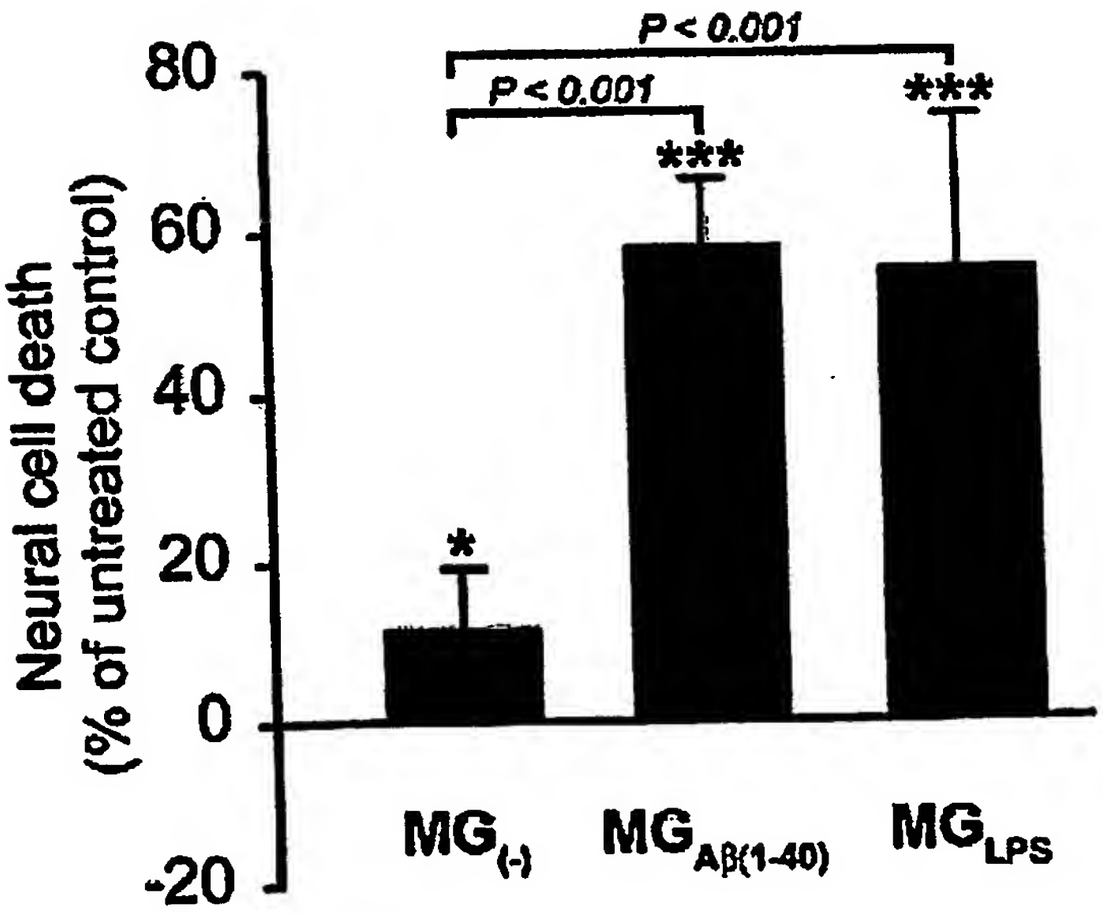
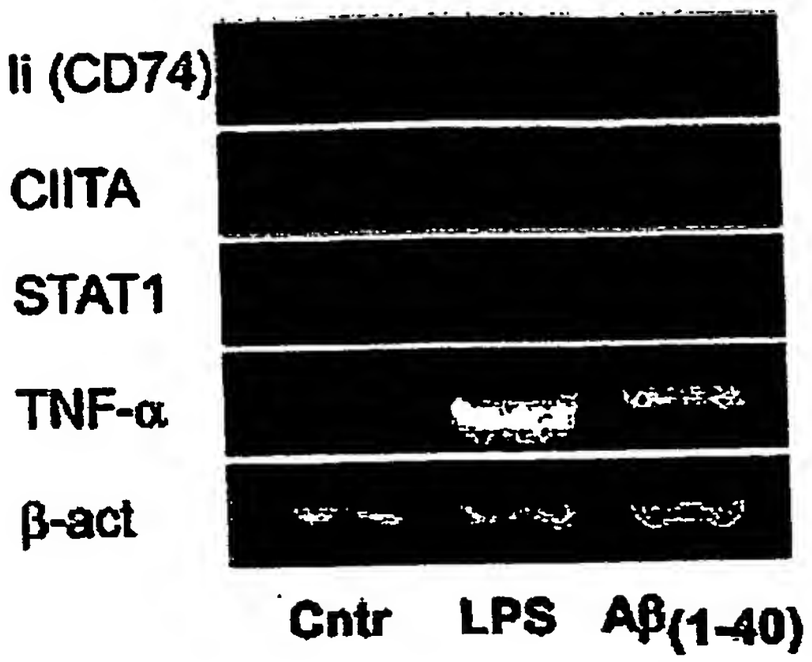

P < 0.001
P < 0.001
***
***
*
Neural cell death
(% of untreated control)
80
60
40
20
0
-20
MG(-)
MGAβ(1-40)
MGLPS

Fig. 8B




**mRNA fold of induction (relative to control)**

| Gene \ Treatments | LPS | $A\beta_{(1-40)}$ |
|---|---|---|
| Ii (CD74) | 1.8±0.1 | 1.5±0.3 |
| CIITA | 1.7±0.2 | 1.2±0.2 |
| STAT1 | 2.4±0.3 | 2.7±0.7 |
| TNF-α | 10.8±0.6 | 6.9±1.6 |

**Fig. 8C**

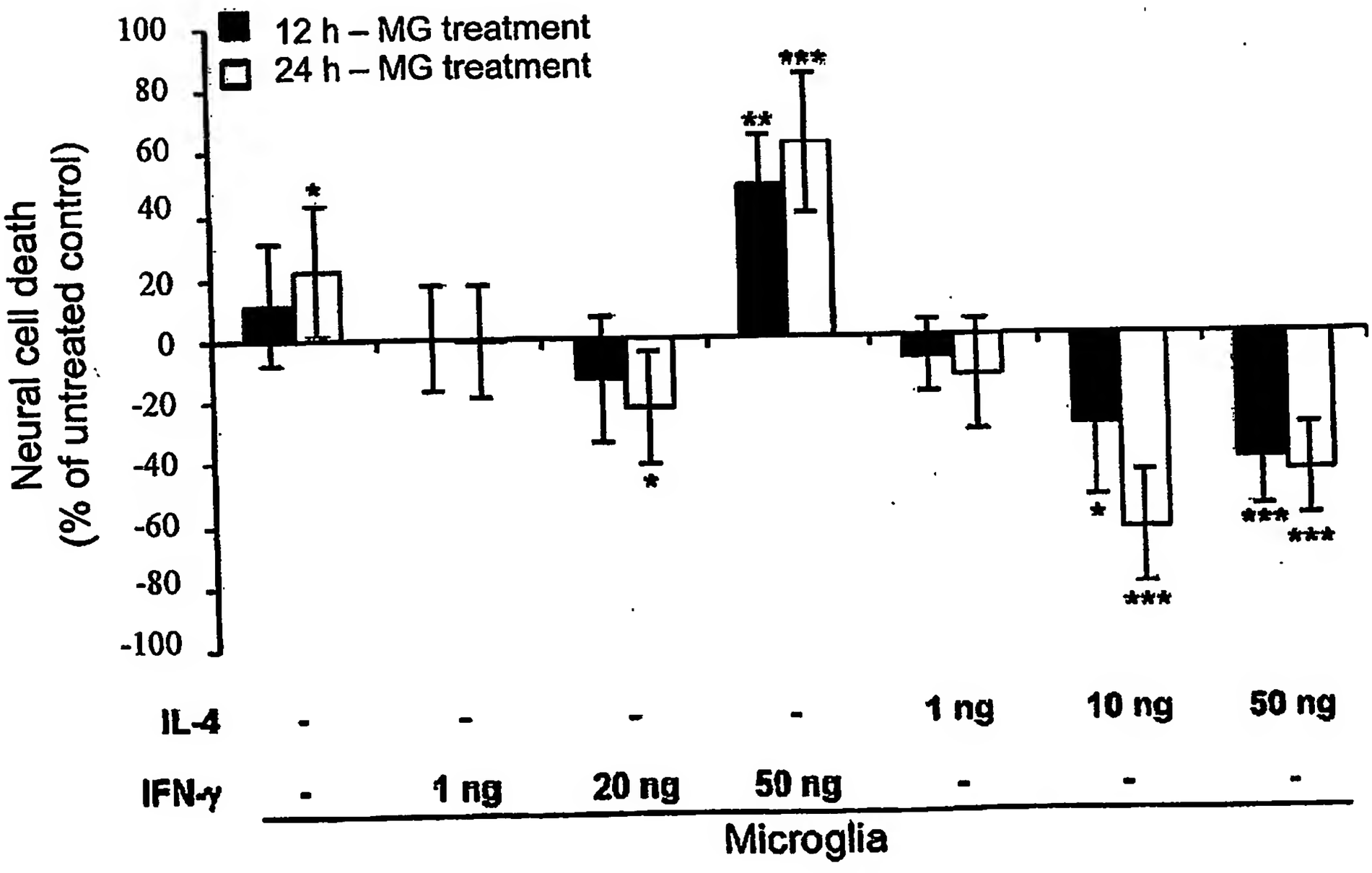
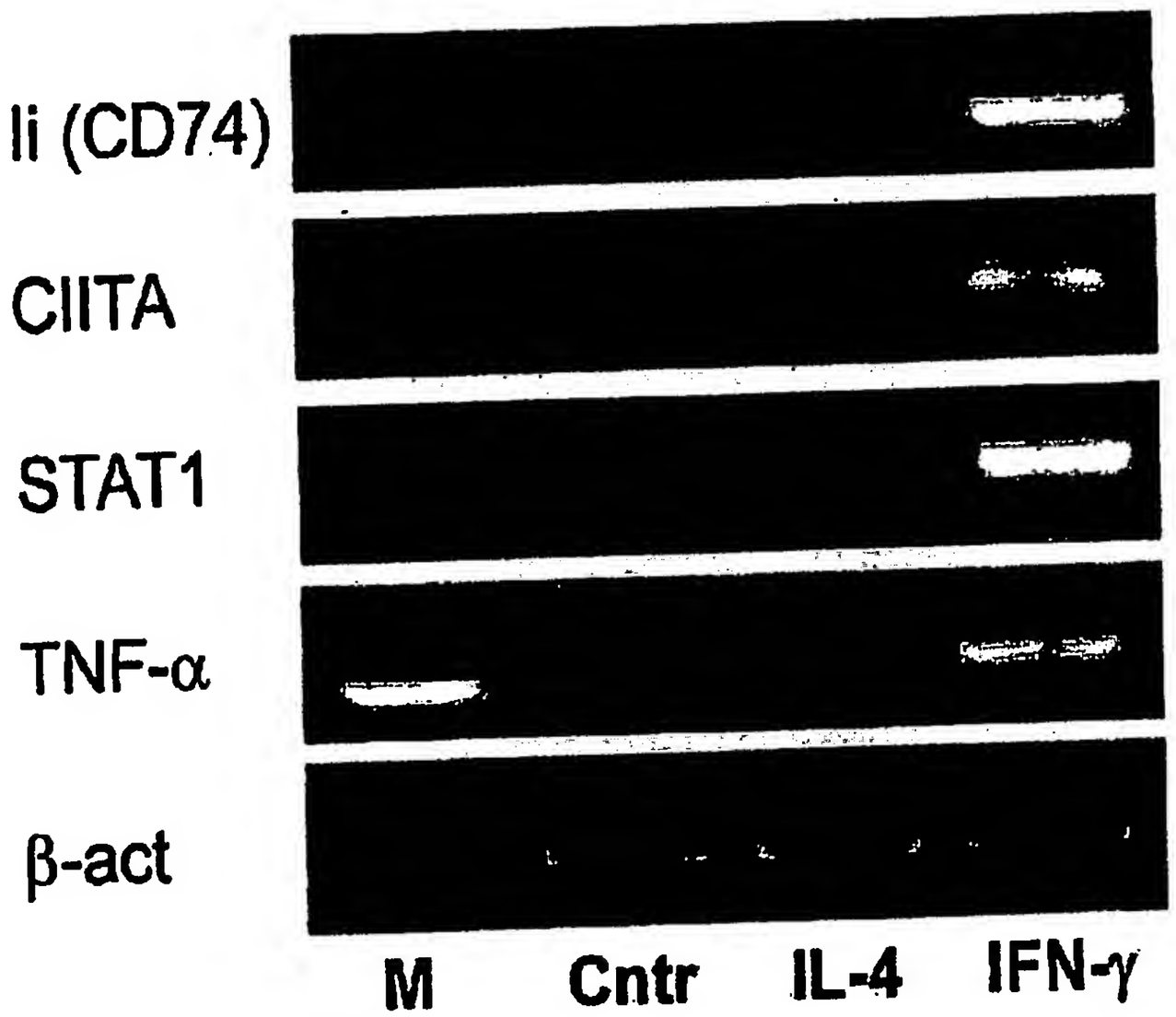



**Fig. 9A**




mRNA fold of induction
(relative to control)

| Treatments / Gene | IL-4 | IFN-γ |
|---|---|---|
| Ii (CD74) | 17.4±4.8 | 52.5±14.6 |
| CIITA | 6.1±0.5 | 40.8±7.2 |
| STAT1 | 15.8±4.2 | 63.3±2.9 |
| TNF-α | 0.5±0.02 | 1.4±0.08 |



**Fig. 9B**

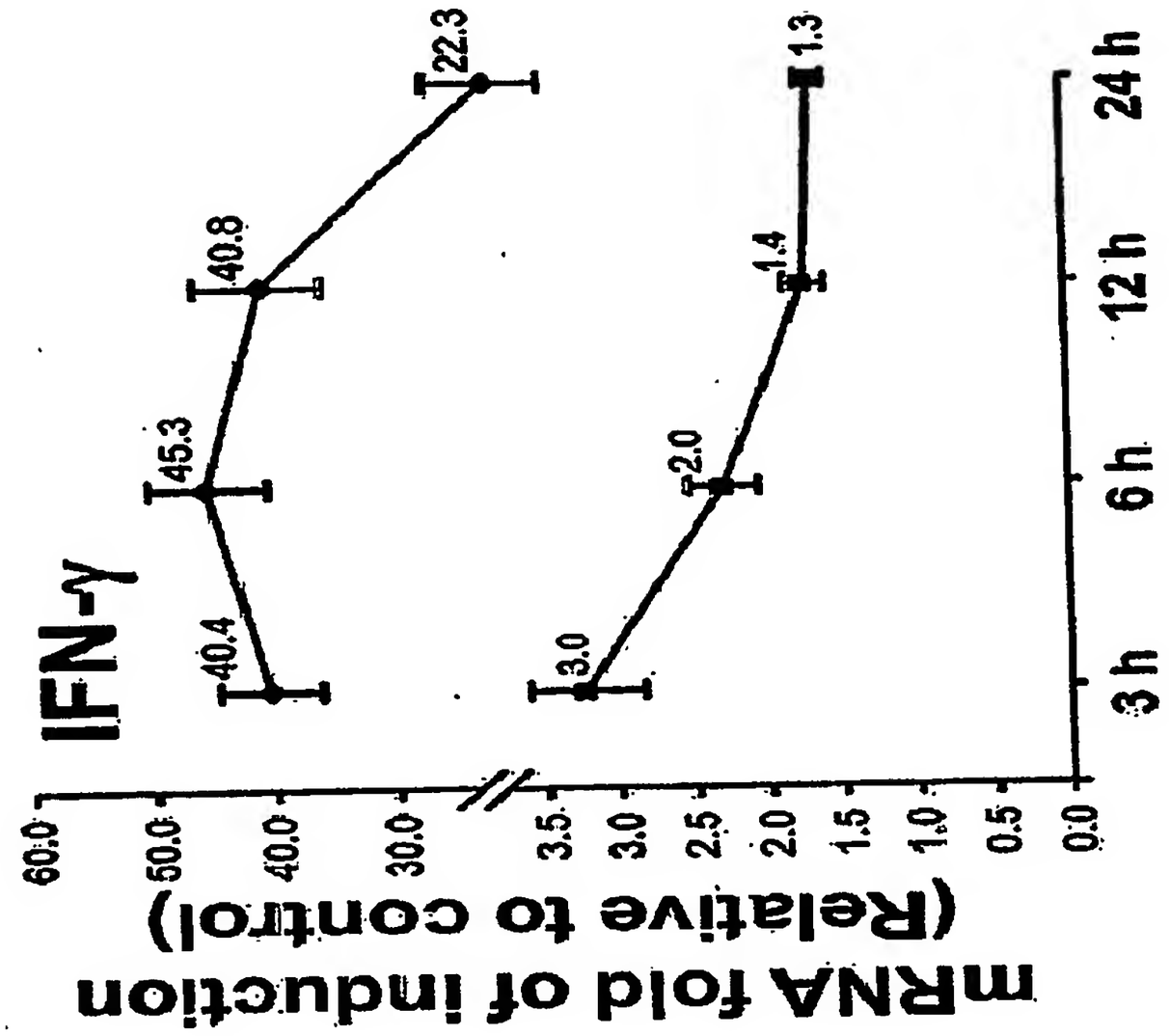
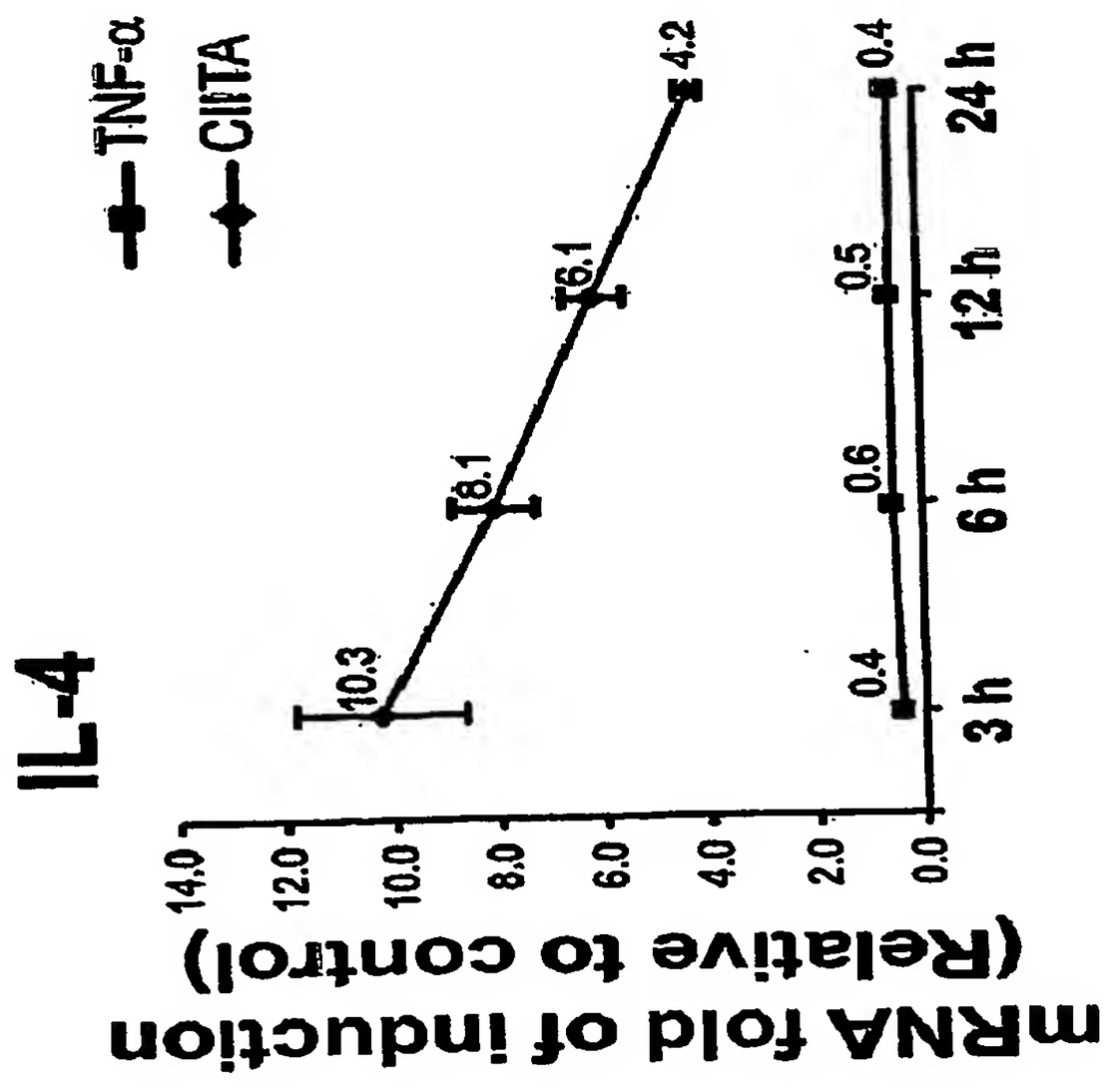

IFN-γ
mRNA fold of induction
(Relative to control)
60.0
50.0
40.0
30.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
40.4
45.3
40.8
22.3
2.0
1.4
1.3
3.0
3 h
6 h
12 h
24 h

Fig. 9C


TNF-α
CIITA
IL-4
mRNA fold of induction
(Relative to control)
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
10.3
T8.1
T6.1
4.2
0.4
0.6
0.5
0.4
3 h
6 h
12 h
24 h

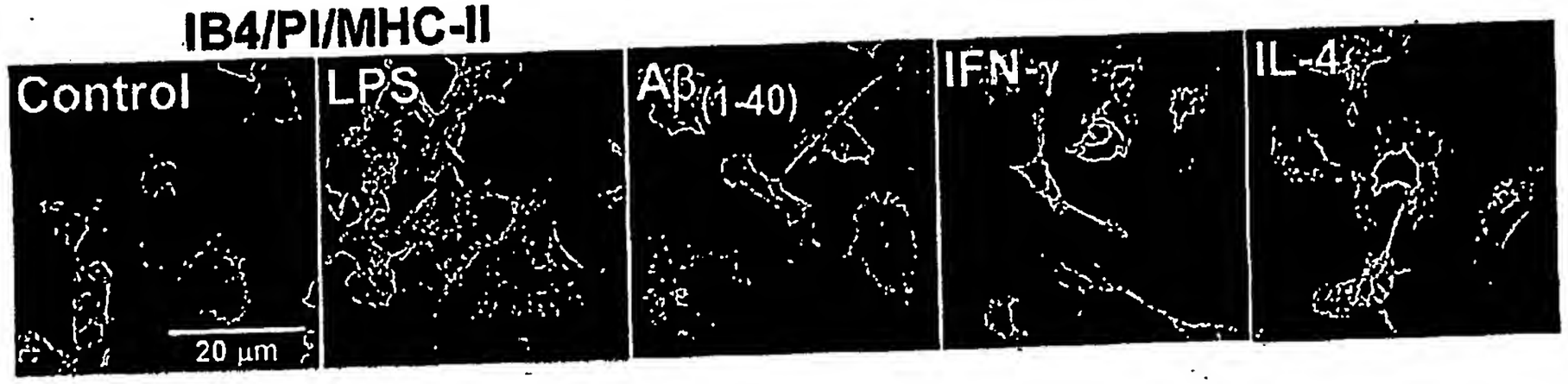



Fig. 10A

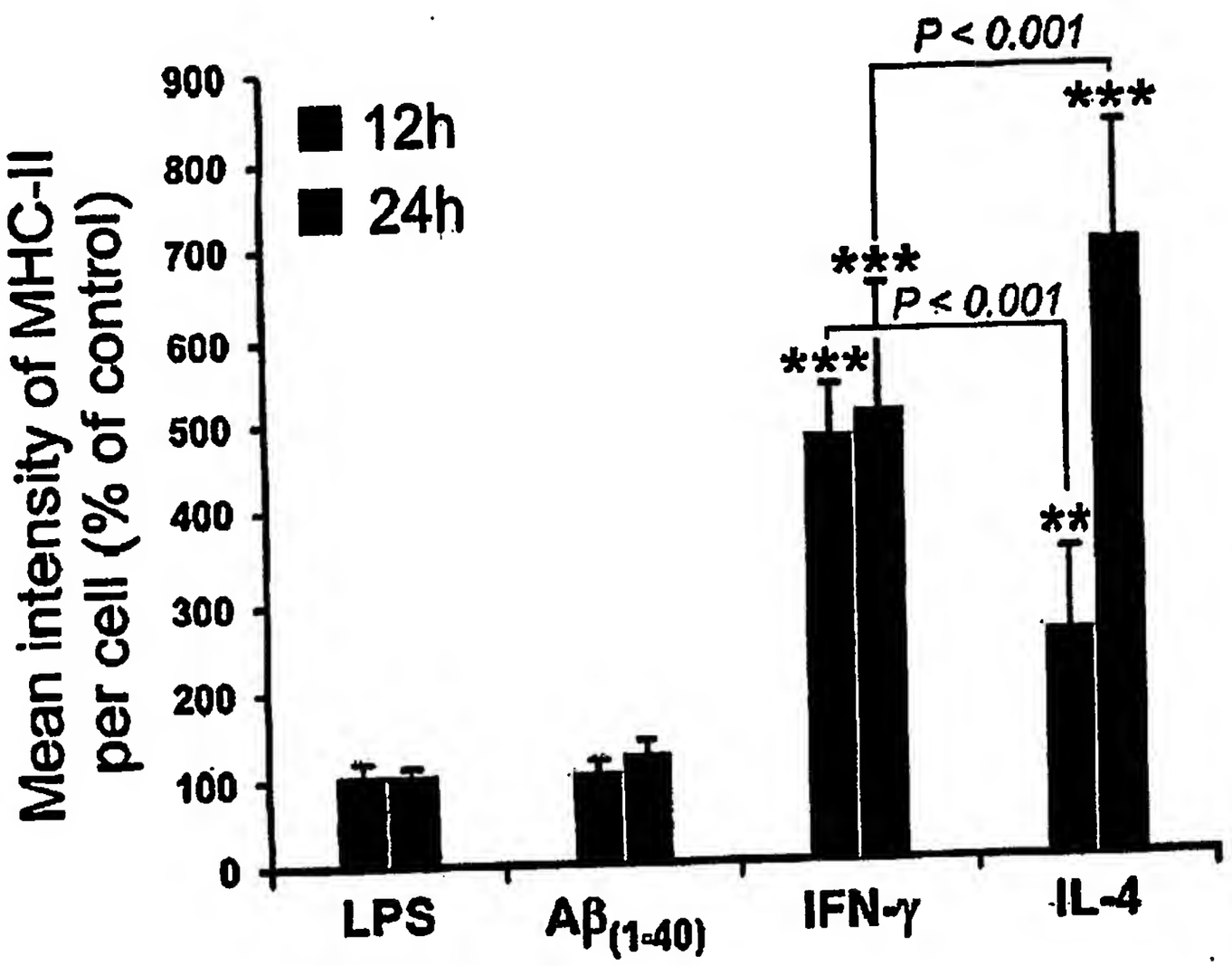



Fig. 10B

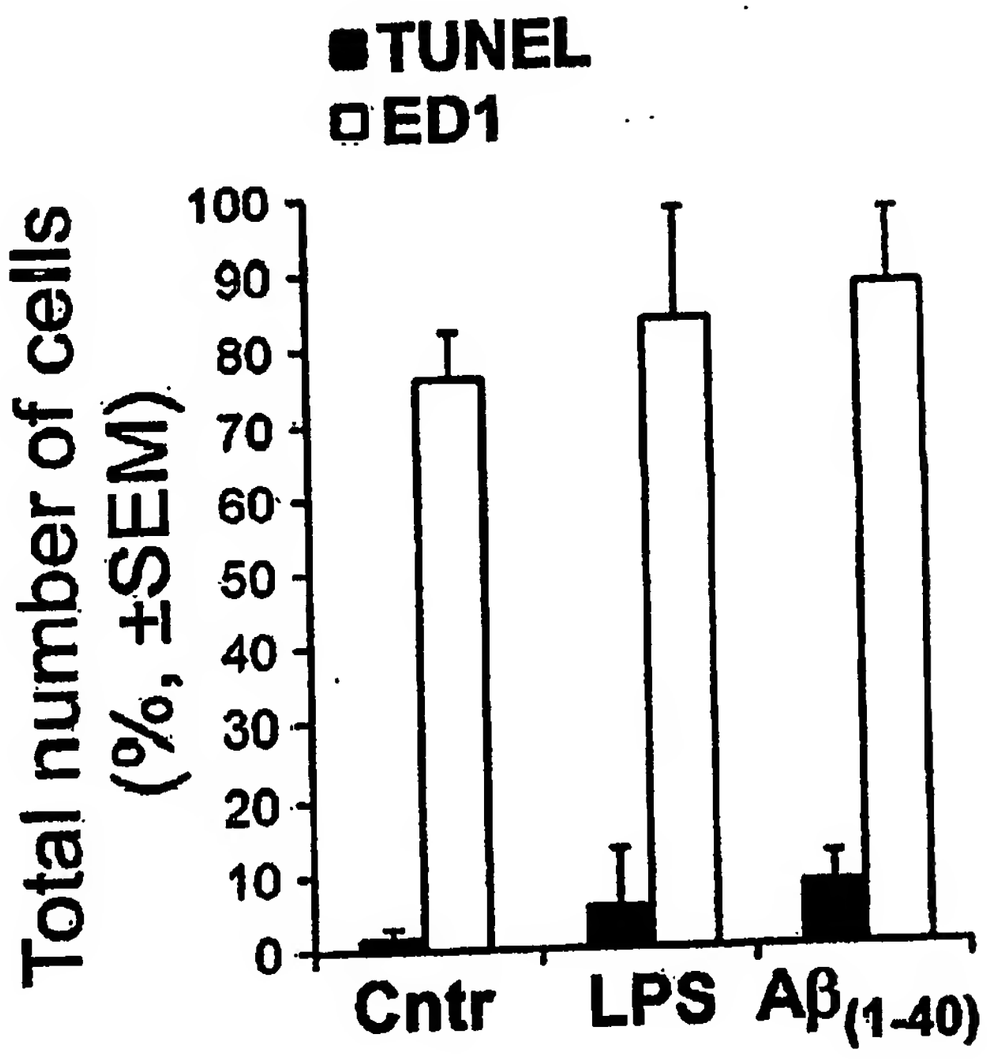



Fig. 10C

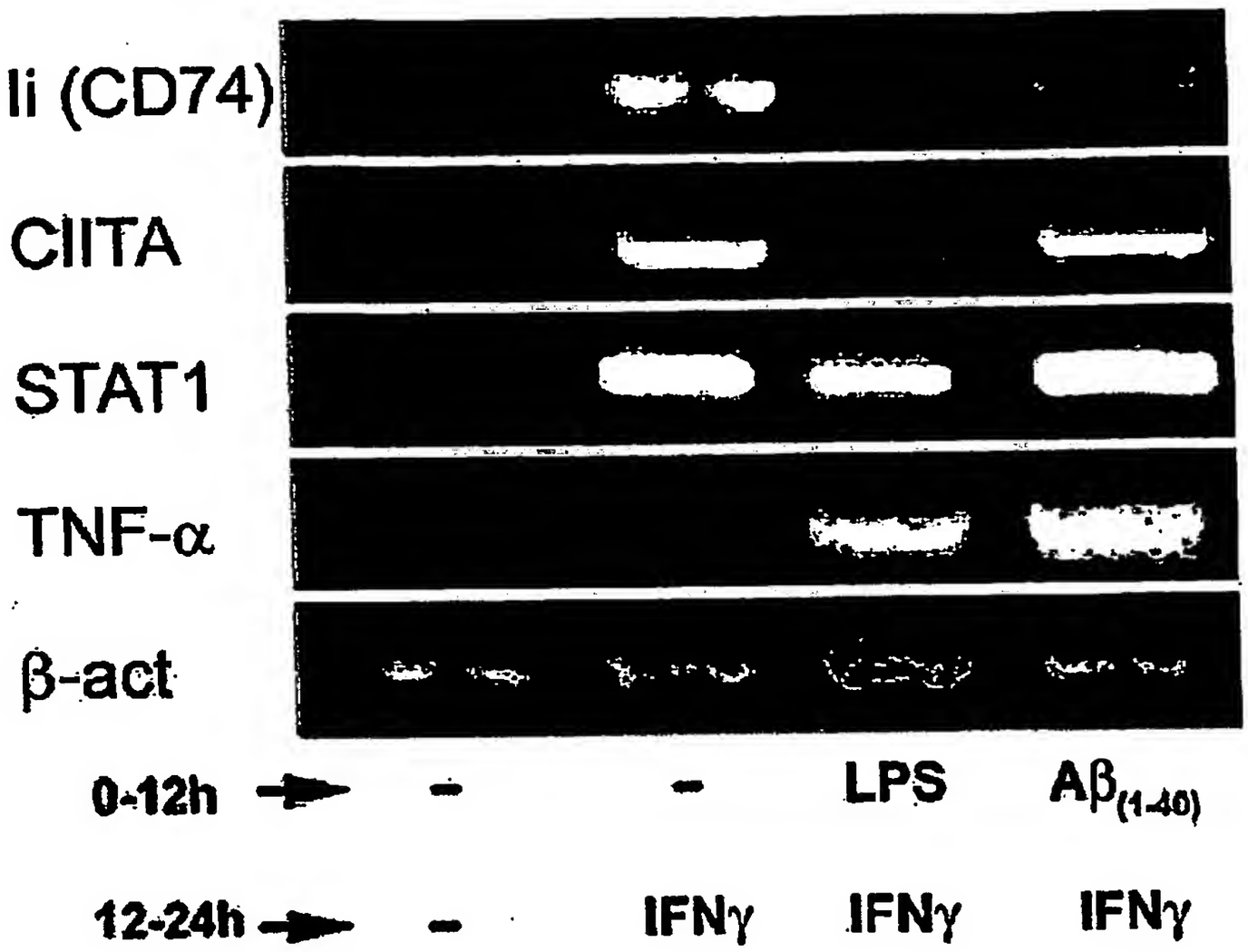



**Fig. 11A**

mRNA fold of induction (relative to control)

| Treatments / Gene | - IFN-γ | LPS IFN-γ | $A\beta_{(1-40)}$ IFN-γ |
|---|---|---|---|
| Ii (CD74) | 52.5±14.6 | 13.4±4.0 | 19.1±4.7 |
| CIITA | 42.4±5.6 | 2.8±0.1 | 37.7±4.6 |
| STAT1 | 63.3±2.9 | 51.5±2.4 | 47.9±3.9 |
| TNF-α | 1.8±0.4 | 10.6±2.8 | 6.7±2.3 |

**Fig. 11B**

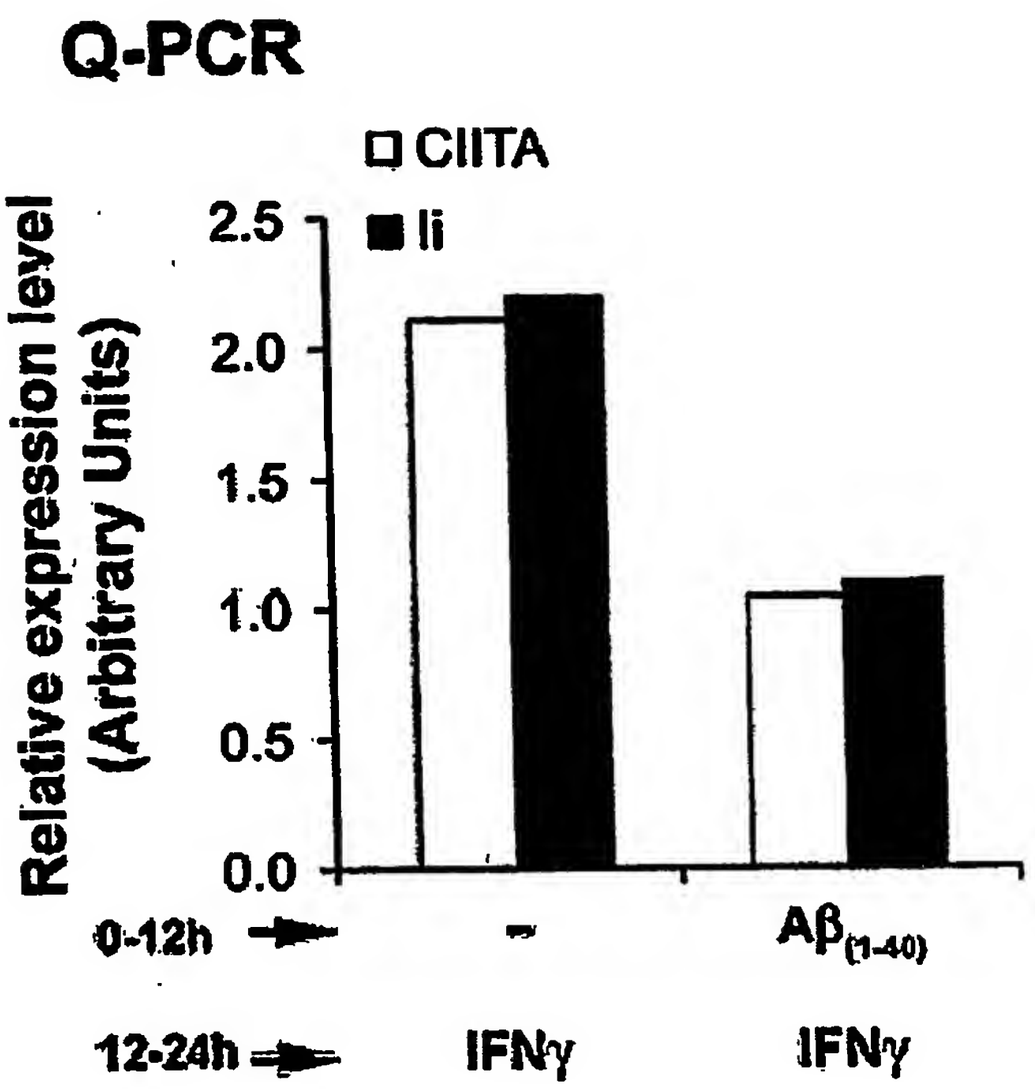



Fig. 11C

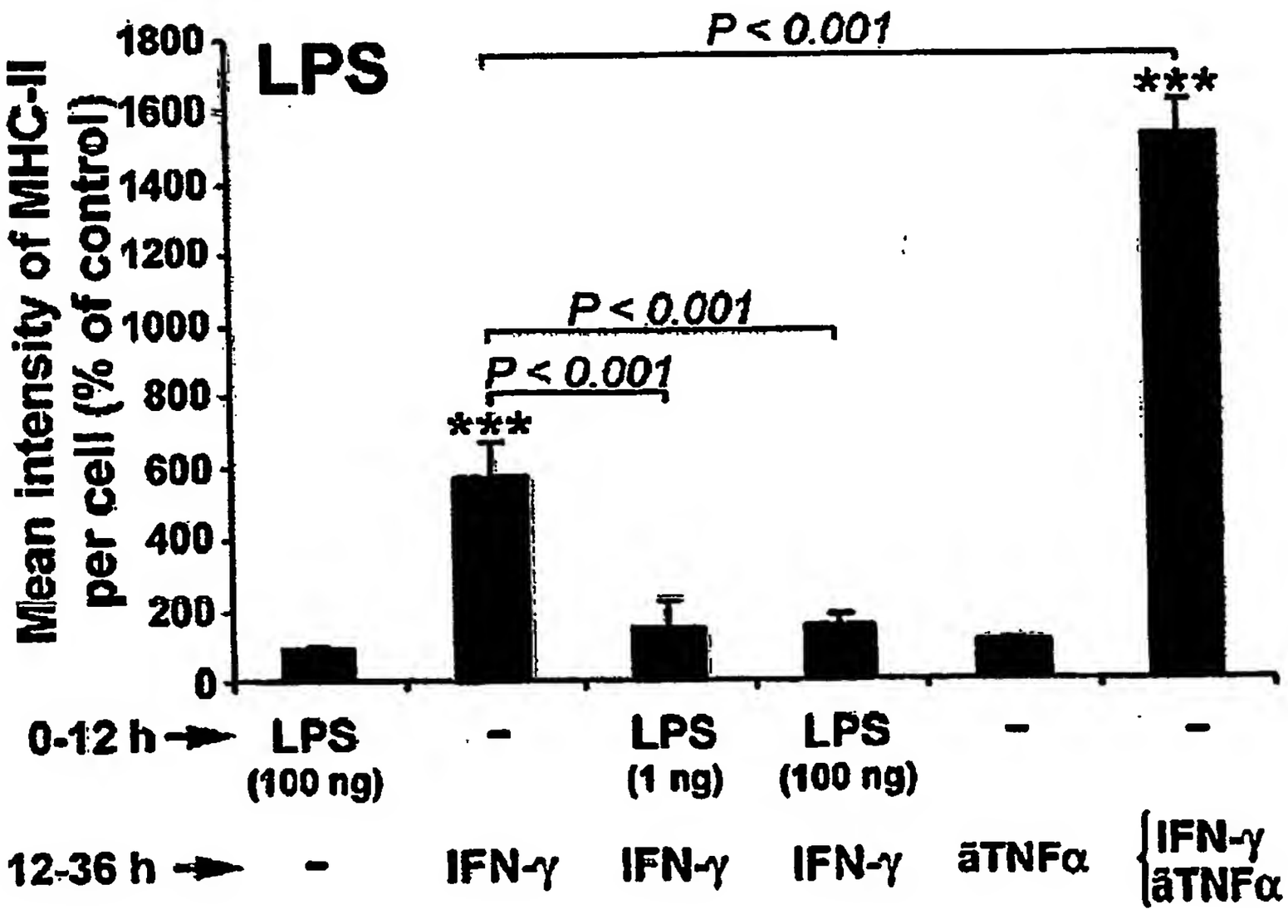



Fig. 11D

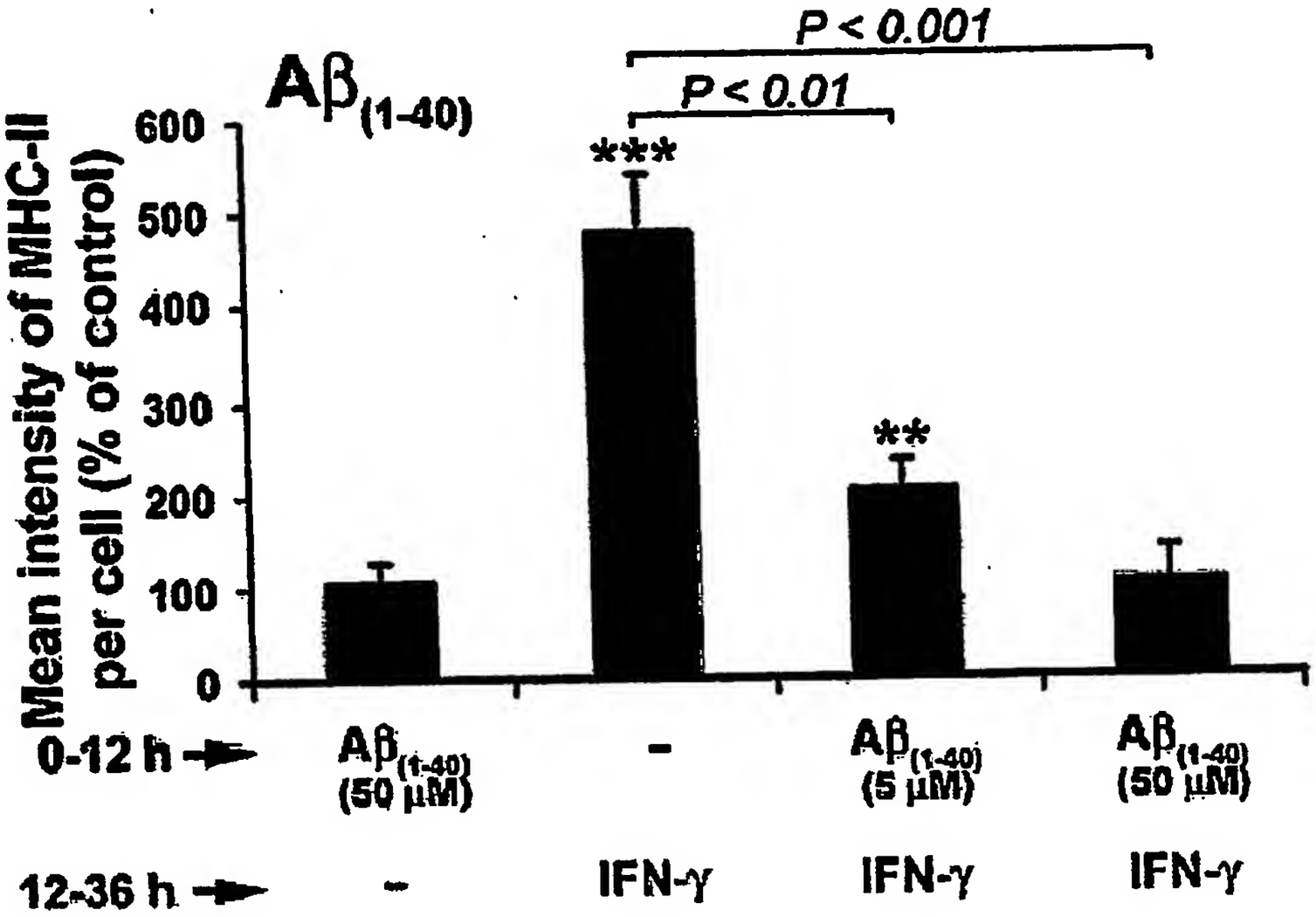



**Fig. 11E**

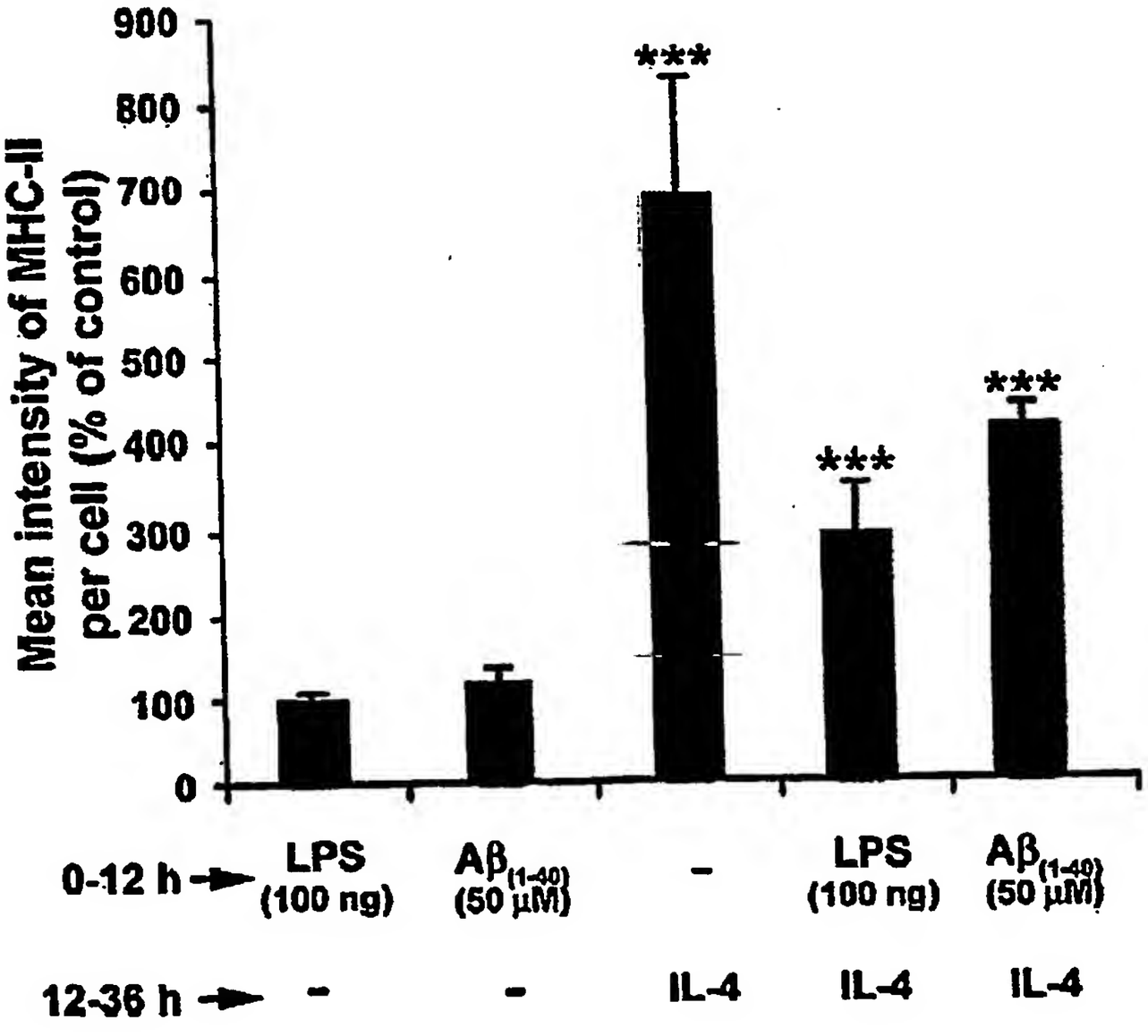



**Fig. 11F**

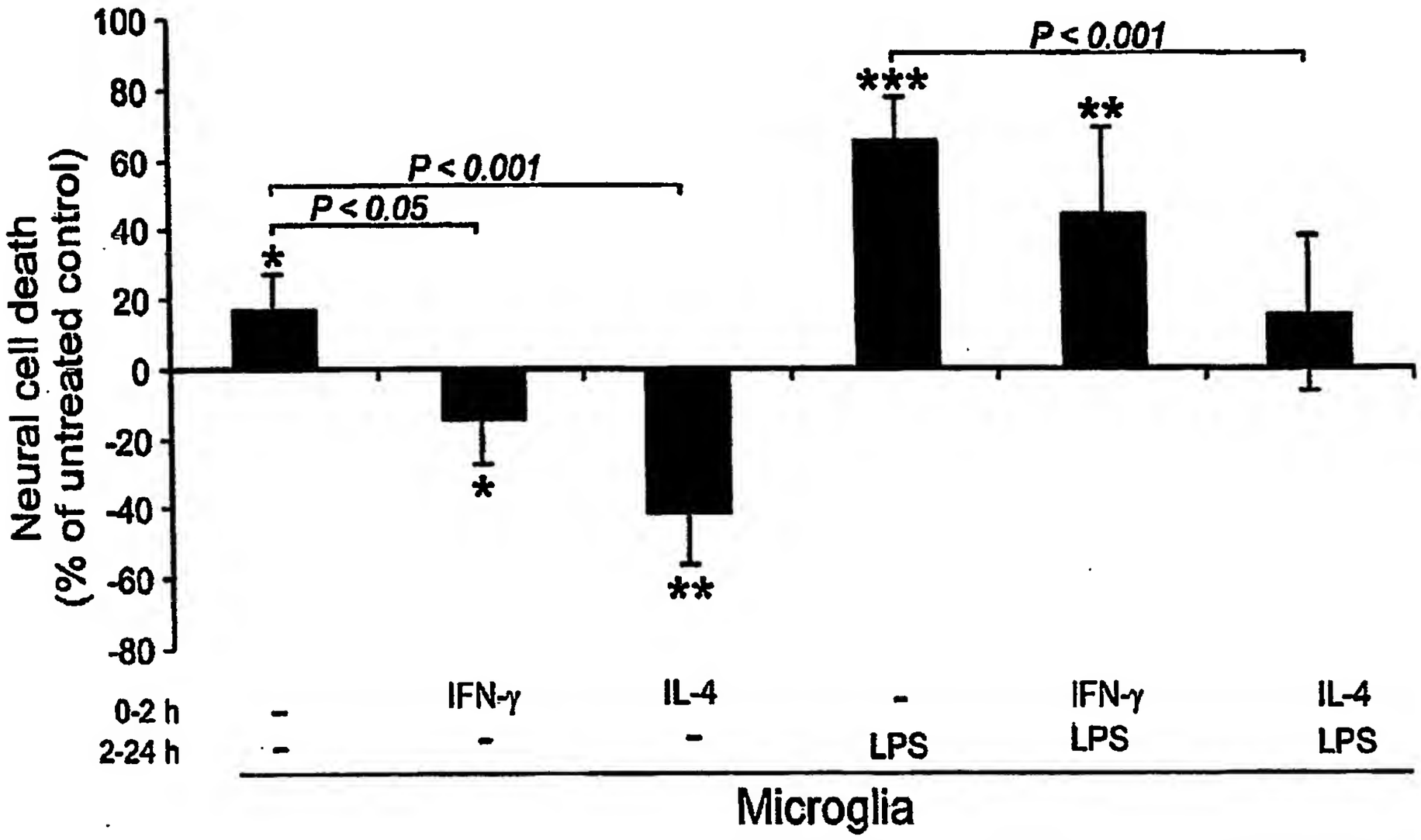
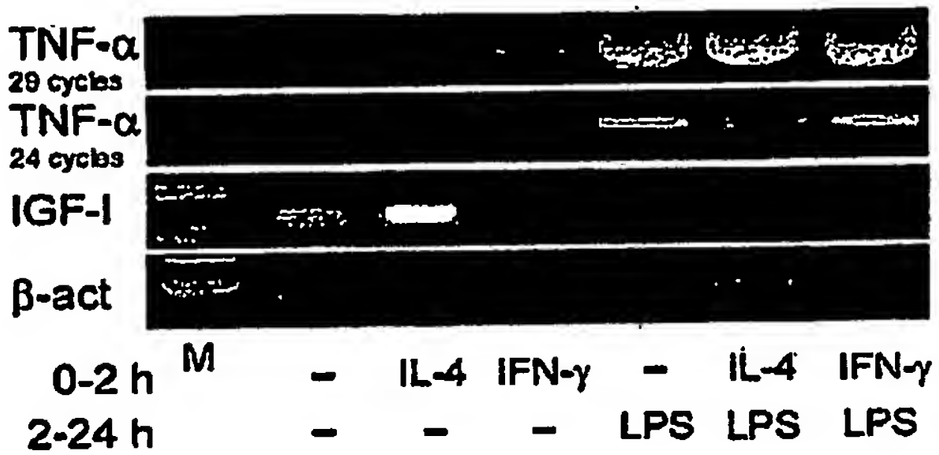
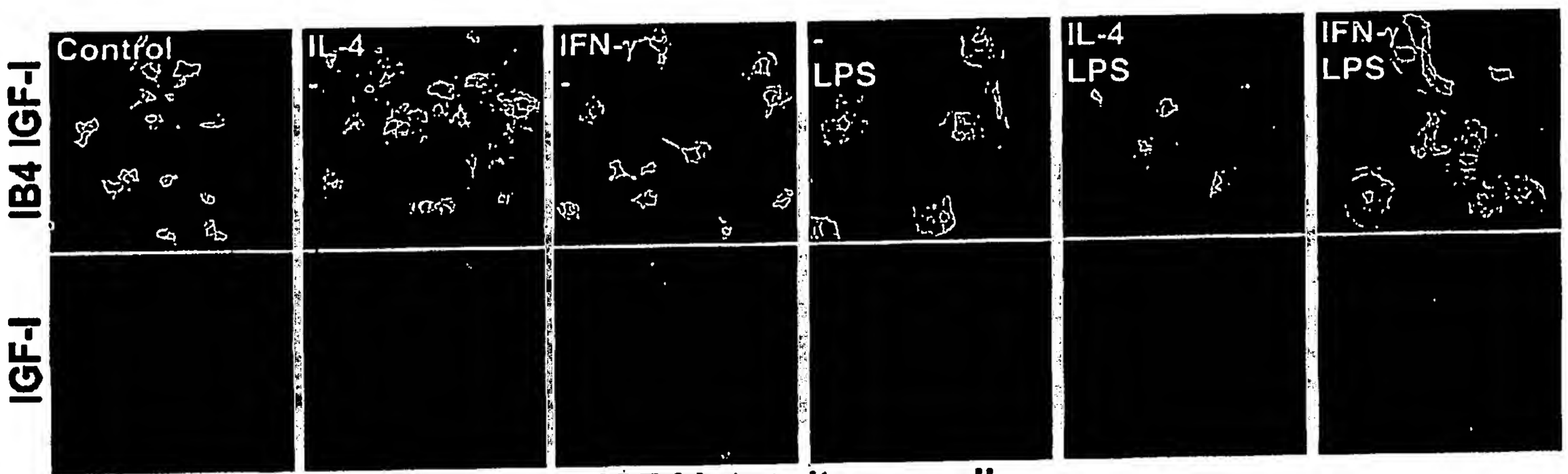

Neural cell death
(% of untreated control)
100
80
60
40
20
0
-20
-40
-60
-80
P < 0.001
P < 0.05
P < 0.001
*
*
**
***
**
0-2 h
2-24 h
-
-
IFN-γ
-
IL-4
-
LPS
IFN-γ
LPS
IL-4
LPS
Microglia

Fig. 12A




| Treatments / Gene | IL-4 – | IFN-γ – | – LPS | IL-4 LPS | IFN-γ LPS |
|---|---|---|---|---|---|
| TNF-α 29 cycles | 0.5±0.02 | 1.3±0.1 | 6.2±1.3 | 6.4±1.1 | 6.5±1.5 |
| TNF-α 24 cycles | – | – | 1.9±0.5 | 1.0±0.05 | 2.2±0.4 |
| IGF-I | 3.5±0.3 | 0.5±0.01 | 0.06±0.01 | 0.4±0.01 | 0.02±0.01 |

**Fig. 12B**

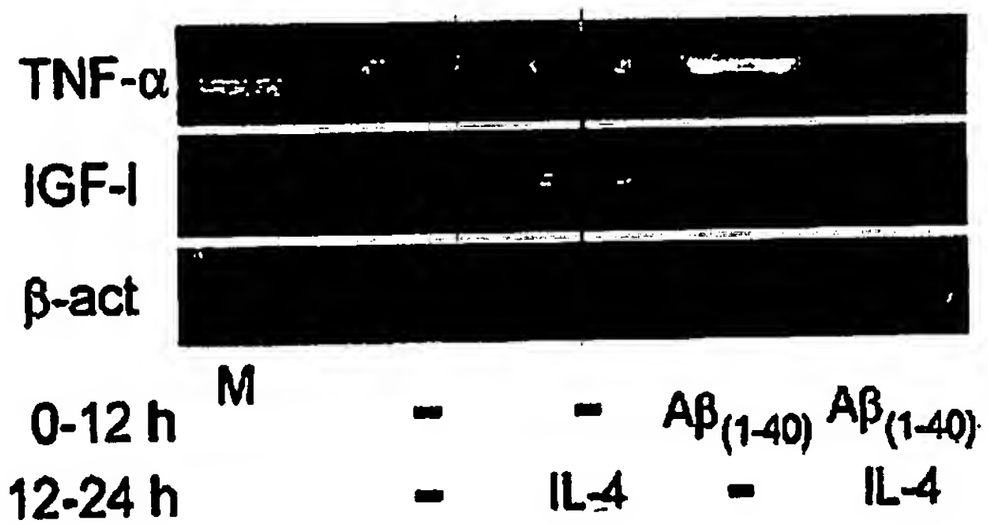



**IGF-I intensity per cell**

| Treatments | IL-4 – | IFN-γ – | – LPS | IL-4 LPS | IFN-γ LPS |
|---|---|---|---|---|---|
| % of control, 100% | 449±95 | 42±23 | 17±13 | 291±72 | 53±39 |
| *P*, Student *t* test | <0.001 | NS | <0.01 | <0.01 | NS |

*Note*.. Results are means ± SEM.. NS, not significant

**Fig. 12C**




mRNA fold of induction (relative to control)

| Treatments / Gene | - IL-4 | $A\beta_{(1-40)}$ - | $A\beta_{(1-40)}$ IL-4 |
|---|---|---|---|
| TNF-α | 0.5±0.02 | 3.6±0.7 | 0.4±0.03 |
| IGF-I | 3.0±0.4 | 0.07±0.02 | 2.6±0.8 |

Fig. 13



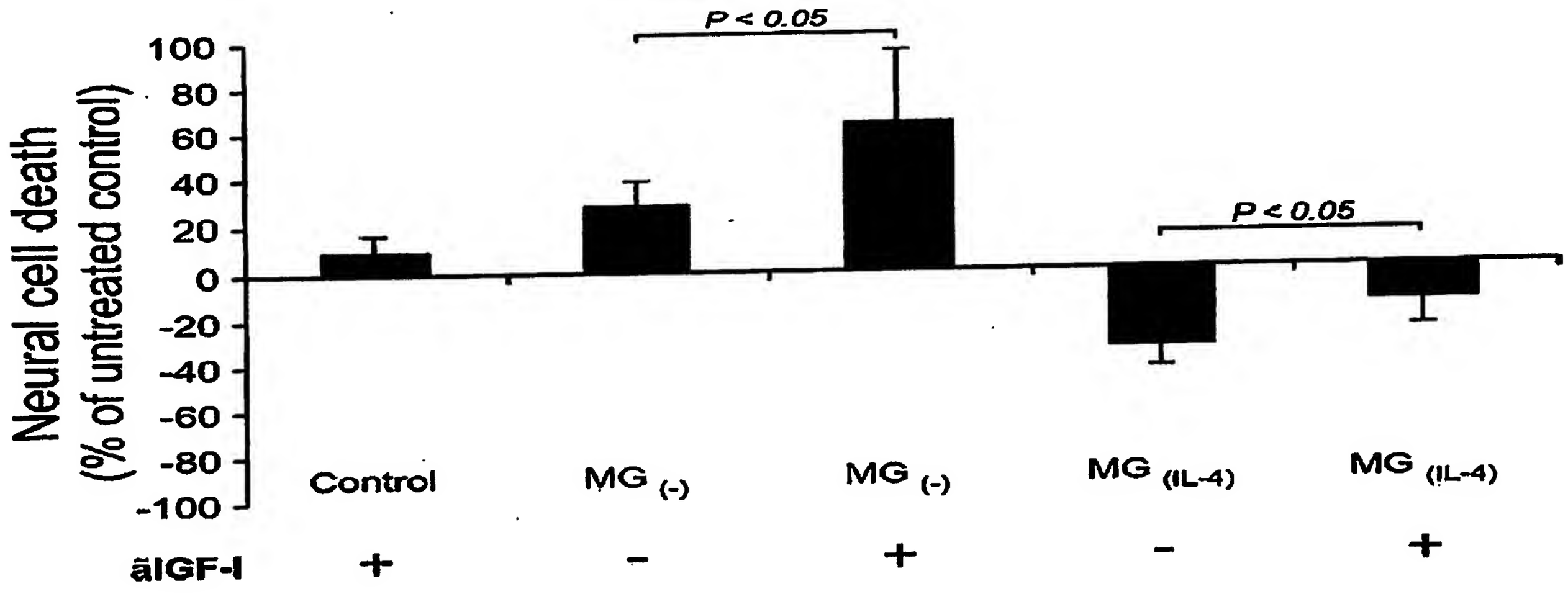



Fig. 14